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December 08, 2004

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APPLICATION NUMBER: *60/517,270*
FILING DATE: *November 04, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/36893*

Certified by

Jon W Dudas

Acting Under Secretary of Commerce
for Intellectual Property
and Acting Director of the U.S.
Patent and Trademark Office

Practitioner's Docket No. **CWR-6622PV** ***PATENT***

Preliminary Classification:

Proposed Class:

Subclass:

Note: *"All applicants are requested to include a preliminary classification on newly filed patent applications. The preliminary classification, preferably class and subclass designations, should be identified in the upper right-hand corner of the letter of transmittal accompanying the application papers, for example 'Proposed Class 2, subclass 129.'" M.P.E.P. § 601, 7th ed.*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: **Jean F. Welter, et al.**

For: **APPARATUS AND METHOD FOR TISSUE ENGINEERING**

Mail Stop: Provisional Patent Application
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

COVER SHEET FOR FILING PROVISIONAL APPLICATION (37 C.F.R. § 1.51(c)(1))

WARNING: *"A provisional application must also include the cover sheet required by § 1.51(c)(1) or a cover letter identifying the application as a provisional application. Otherwise, the application will be treated as an application filed under paragraph (b) [nonprovisional application] of this section." 37 C.F.R. § 1.53(c)(1). See also M.P.E.P. § 201.04(b), 6th ed,. rev. 3.*

CERTIFICATE OF MAILING/TRANSMISSION 37 CFR 1.8(a) and 1.10*
(When using Express Mail, the Express Mail label number is mandatory; Express Mail Certification is optional.)

I hereby certify that, on the date shown below, this correspondence is being:

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☒ deposited with the United States Postal Service in an envelope addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

37 C.F.R. § 1.8(a)

☐ with sufficient postage as first class mail.

37 C.F.R. § 1.10*

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TRANSMISSION

☐ transmitted by facsimile to the Patent and Trademark Office, (703) _____.

_____
Signature

Date: November 4, 2003

Leslie Ann Kuder
(type or print name of person certifying)

**Only the date of filing (§1.6) will be the date used in a patent term adjustment calculation, although the date on any certificate of mailing or transmission under § 1.8 continues to be taken into account in determining timeliness. See §*

1.703(f). Consider "Express Mail Post Office Addressee" (§ 1.10) or facsimile transmission (§ 1.6(d)) for the reply to be accorded to the earliest possible filing date for patent term adjustment calculations.

NOTE: "A complete provisional application does not require claims since no examination on the merits will be given to a provisional application. However, provisional applications may be filed with one or more claims as part of the application. Nevertheless, no additional claim fee or multiple dependent claims fee will be required in a provisional application." Notice of December 5, 1994, 59 Fed. Reg. 63,951, at 63,953. "Any claim filed with a provisional application will, of course, be considered part of the original provisional application disclosure." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.

NOTE: "A provisional application is not entitled to the right of priority under 35 U.S.C. 119 or 365(a) or § 1.55, or to the benefit of an earlier filing date under 35 U.S.C. 120, 121 or 365(c) or § 1.78 of any other application. No claim for priority under § 1.78(a)(3) may be made in a design application based on a provisional application. No request under § 1.293 for a statutory invention registration may be filed in a provisional application. The requirements of §§ 1.821 through 1.825 regarding application disclosures containing nucleotide and/or amino acid sequences are not mandatory for provisional applications." 37 C.F.R. § 1.53(c)(3).

NOTE: "No information disclosure statement may be filed in a provisional application." 37 C.F.R. § 1.51(d). "Any information disclosure statements filed in a provisional application would either be returned or disposed of at the convenience of the Office." Notice of December 5, 1994, 59 Fed. Reg. 63,591, at 63,594.

NOTE: "No amendment other than the provisional application comply with the patent statute and all applicable regulations may be made to the provisional application after the filing date of the provisional application." 37 C.F.R. § 1.53(c).

NOTE: 35 U.S.C. 119(e) provides that "[i]f the day that is 12 months after the filing date of a provisional application falls on a Saturday, Sunday, or Federal Holiday within the District of Columbia, the period of pendency of the provisional application shall be extended to the next succeeding secular or business day ".

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.51(c)(1)(i).

1. The following comprises the information required by 37 C.F.R. § 1.51(c)(1):

2. The name(s) of the inventor(s) is/are (37 C.F.R. § 1.51(c)(1)(ii):

NOTE: "If the correct inventor or inventors are not named on filing a provisional application without a cover sheet under § 1.51(c)(1), the later submission of a cover sheet under § 1.51(c)(1) during the pendency of the application will act to correct the earlier identification of inventorship." 37 C.F.R. § 1.48(f)(2).

NOTE: "The naming of inventors for obtaining a filing date for a provisional application is the same as for other applications. A provisional application filed with the inventors identified as 'Jones et al.' will not be accorded a filing date earlier than the date upon which the name of each inventor is supplied unless a petition with the fee set forth in § 1.17(i) is filed which sets forth the reasons the delay in supplying the names should be excused. Administrative oversight is an acceptable reason. It should be noted that for a 35 U.S.C. 111(a) application to be entitled to claim the benefit of the filing date of a provisional application the 35 U.S.C. 111(a)[,] application must have at least one inventor in common with the provisional application." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.

The term "invention" is typically used to refer to subject matter which applicant is claiming in his/her application. Because claims are not required in a provisional application, it would not be appropriate to reference joint inventors as those who have made a contribution to the "invention" disclosed in the provisional application. If the "invention" has not been determined in the provisional application because no claims have been presented, then the name(s) of those person(s) who have made a contribution to the subject matter disclosed in the provisional application should be submitted. Section 1.45(c) states that "if multiple inventors are named in a provisional application, each named inventor must have made a contribution, individually or jointly, to the subject matter disclosed in the provisional application." All that § 1.45(c) requires is that if someone is named as an inventor, that person must have made a contribution to the subject matter disclosed in the provisional application. When applicant has determined what the invention is by the filing of the 35 U.S.C. 111(a) application, that is the time when the correct inventors must be named. The 35 U.S.C. 111(a) application must have an inventor in common with the provisional application in order for the 35 U.S.C. 111(a) application to be entitled to claim the benefit of the provisional application under 35 U.S.C. 119(e). Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,208.

See 37 C.F.R. § 1.53.

	GIVEN NAME	MIDDLE INITIAL OR NAME	FAMILY (OR LAST) NAME
1.	Jean	F.	Welter
2.	Luis	A.	Solchaga
3.	Jim	A.	Berilla

IVEN NAME — MIDDLE INITIAL R NAME — FAMILY (R LAST) NAME

4. Kitsie | | Penick
GIVEN NAME | MIDDLE INITIAL R NAME | FAMILY (R LAST) NAME

3. Residence address(es) of the inventor(s), as numbered above (37 C.F.R. §1.51(c)(1)(iii):

1. 17933 Sherrington Road, Shaker Heights, Ohio 44122
2. 2260 Barrington Road, University Heights, Ohio 44188
3. 535 Ransome Road, Highland Heights, Ohio 44143
4. 798 Tote Road, Austinburg, Ohio 44010

4. The title of the invention is (37 C.F.R. § 1.51(c)(1)(iv)):

APPARATUS AND METHOD FOR TISSUE ENGINEERING

5. The name, registration, customer and telephone numbers of the practitioner (if applicable is (37 C.F.R. § 1.51(c)(1)(v)):

Name of practitioner: Richard A. Sutkus

Reg. No.:43,941 Tel. (216) 621-2234

Customer No. 26,294

(complete the following, if applicable)

☐ A power of attorney accompanies this cover sheet.

6. The docket number used to identify this application is (37 C.F.R. §1.51(c)(1)(vi)):
Docket No.: CWR-6622PV

7. The correspondence address for this application is (37 C.F.R. §1.51(c)(1)(vii)):

TAROLLI, SUNDHEIM, COVELL, & TUMMINO L.L.P.
526 SUPERIOR AVENUE, Suite 1111
CLEVELAND, OH 44114-1400

8. Statement as to whether invention was made by an agency of the U.S. Government or under contract with an agency of the U.S. Government. (37 C.F.R. § 1.51(c)(1)(viii)).

This invention was made by an agency of the U.S. Government, or under contract with an agency of the U.S. Government.

☒ No.

☐ Yes.

The name of the U.S. Government agency and the Government contract number are:

9. Identification of documents accompanying this cover sheet.

A. Documents required by 37 C.F.R. §§ 1.51(c)(2)-(3):

Specification: No. of pages **36**

Drawings: No. of sheets **30**

B. Additional documents:

☒ Claims: No. of claims

☒ Abstract No. of pages 2

Note: See 37 C.F.R. § 1.51.

☐ Power of attorney

☐ Small entity statement

☐ Assignment

☐ Other:

NOTE: Provisional applications may be filed in a language other than English as set forth in existing § 1.52(d). However, an English language translation is necessary for security screening purposes. Therefore, the PTO will require the English language translation and payment of the fee mandated in § 1.52(d) in the provisional application. Failure to timely submit the translation in response to a PTO requirement will result in the abandonment of the provisional application. If a 35 U.S.C. 111(a) application is filed without providing the English language translation in the provisional application, the English language translation will be required to be supplied in every 35 U.S.C. 111(a) application claiming priority of the non-English language provisional application. Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.

10. Fee

The filing fee for this provisional application, as set in 37 C.F.R. § 1.16(k), is $160.00, for other than small entity, and $80.00, for a small entity.

☒ Applicant is a small entity.

NOTE: "A . . . statement in compliance with existing § 1.27 is required to be filed in each provisional application in which it is desired to pay reduced fees." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,197.

11. Small entity statement assertion

☐ The assertion that this is a filing by a small entity under 37 C.F.R. § 1.27 (c)(1) is attached ("ASSERTION OF SMALL ENTITY STATUS").

☒ Small entity status is asserted for this application by payment of the small entity filing fee under § 1.16(k). 37 C.F.R. § 1.27(c)(3).

12. Fee payment

☒ Fee payment in the amount of **$80.00** is being made at this time.

☐ No filing fee is to be paid at this time (This and the surcharge required by 37 C.F.R. § 1.16(l) can be paid subsequently).

13. Method of fee payment

☒ Attached is a ☒ check ☐ money order in the amount of **$80.00**.

☐ Authorization is hereby made to charge the amount of $_____.

☐ to Deposit Account No. 20-090.

☐ to Credit card as shown on the attached credit card information authorization form PTO-2038.

WARNING: *Credit card information should **not be** included on this form as it may become public.*

☒ Charge any additional fees required by this paper or credit any overpayment in the manner authorized above.

A duplicate of this paper is attached.

Date: November 4, 2003

Signature of practitioner

Reg. No. 43,941

Richard A. Sutkus
(type or print name of practitioner)

Tel. No. (216) 621-2234

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APPARATUS AND METHOD FOR TISSUE ENGINEERING

TECHNICAL FIELD

The present invention relates generally to apparatuses and methods for tissue engineering, and more particularly, to apparatuses and methods for generating tissue engineered constructs.

BACKGROUND OF THE INVENTION

The culturing of living cells *in vitro* is performed for a variety of purposes, including the preparation of viral vaccines, the recovery of by-products of cell metabolism, and the production of tissue or tissue-like derivatives for creating implants or artificial organs. Cell types that can be grown in culture can include connective tissue cells, skeletal cells, cardiac cells, epithelial cells, neural cells, endocrine cells, melanocytes, and many types of tumor cells. A variety of media are available, depending on the particular growth requirements of the cells and the growth conditions.

Typically, cell culture production of either cells or cell-secreted products begins with the small scale growth of cells. Traditional vessels for small volume cultures include multi-well plates, T-flasks, roller bottles and spinner flasks. In recent years a number of manufacturers have also begun to offer cell culture devices in the form of flexible, disposable bags formed of biologically inert and gas-permeable plastic materials such as fluoro-ethylene-propylene copolymers. Cell culture devices that involve the use of oxygen-permeable materials have also been used in recent years. For instance, a cell culture device can be constructed from a petri-dish that includes a base, which consists of a gas-permeable membrane, such as silicone rubber. Alternatively, a cell culture device can be constructed from a Roux bottle in which a side wall comprises the gas-permeable membrane.

Several problems are associated with growing cells *in vitro* to produce dense masses of cells. First, individual components of the nutrient medium must diffuse through the cell layers to reach all cells. This becomes increasingly difficult as the thickness of the cell layer increases. Second, the maintenance of a suitable environment for cell growth is difficult because the fluid immediately adjacent a growing cell is

continuously changing as cellular metabolism proceeds and is returned to its original status only in stepwise fashion when the nutrient medium is changed or agitated *en masse*. Third, a lattice or suitable material upon which to grow some types of cells is often required.

SUMMARY OF THE INVENTION

The present invention relates to apparatuses and methods for tissue engineering. More particularly, the present invention relates to apparatuses and methods for generating tissue engineered constructs, such as tissue engineered cartilage implants for articular cartilage repair. It will be appreciated by one skilled in the art that the apparatuses and methods of the present invention can be used for generating other cell or tissue constructs, such as tissue engineered skin, tissue engineered bone, and tissue engineered tendon.

In accordance with one aspect of the invention, tissue engineered constructs can be grown in a bioreactor system that can provide continuous perfusion of a cell culture medium to help improve mass transfer rates of cells of the tissue engineered constructs. The bioreactor system can provide multiple stimuli to the cells simultaneously, and can be instrumented for diagnostic measurements. The bioreactor system can be modular and comprise two outer rigid frames (*e.g.*, aluminum) and a biocompatible inner frame (*e.g.*, Delrin®), which is capable of being sterilized. The inner frame can define a culture chamber that can contain cell culture media. The inner frame can include media inlet and outlet ports. Additional ports can be provided for instrumentation, sample collection, and media recirculation. The bioreactor system can further include membranes that can be used to the seal the chamber. The membranes can include biocompatible and sterilizable gas permeable materials (*e.g.*, fluoro-ethylene-propylene (Teflon®)) that can be used for gas exchange with the culture chamber. The membranes can be rigid to protect tissue engineered construct cultured in the chamber from the hydrostatic pressure used for gas exchange. The membranes can also comprise substantially non-gas permeable materials. The membranes can be sealed with gaskets (*e.g.*, silicone) to the inner frame.

The chamber can form the core of the modular system to which can be fitted, for example, pH measurement instrumentation, hydrostatic mechanical stimulation modules,

oxygen probes, CO_2 probes, glucose sensors, and other sensors for measuring selected products or conditions. Monitoring systems can be set to clamp selected environmental parameters at predetermined values, while allowing others to float as outcome variables, and can be set to flag specific conditions as requiring intervention.

The bioreactor system can be designed as a self-contained system, which, after being assembled in a sterile environment, does not need to be opened until the end of the experimental run. The chamber can be perfused with cell culture medium using a microprocessor controlled syringe pump. The medium can be collected in waste containers and not recycled. The flow-through approach avoids intermittent drastic changes in the extracellular environment associated with bulk medium exchanges, and can allow the establishment of a stable extracellular environment.

Another aspect of the invention relates to a multi-channel fiber-optic spectrophotometer. The spectrophotometer can measure light transmission and absorbance of at least one molecular species in at least one sample. For example, the spectrophotometer can be used to continuously measure the pH of the cell culture medium at an inlet and an outlet of a culture chamber. In this example, the spectrophotometer can capture the phenol red absorption spectra at 559 nm. The spectrophotometer can include a monochromator that can be modified to position polished ends of optical fibers (*e.g.*, 500 μm) in an output slit plane. The wavelength can be adjusted using a stepper motor (40 steps/nm). Flow-through cuvettes can be positioned at the inlet and outlet of each culture chamber. Cuvette holders can align the collimated output from each fiber, the cuvette, and a detector-amplifier hybrid. Data collection and control can be synchronized using custom software. The correlation (r^2 = 0.998) between pH and transmission at 559 nm in the physiologically relevant range permits calibration of the device in pH units. pH differences of 0.05 units were readily resolved. Better resolution can be expected with sampling at more wavelengths. Repeat measurements of the same samples correlated with an r^2 of better than 0.99999.

A further aspect of the invention relates to a method of improving chondrogenesis by adult mesenchymal stem cells. In the method, a suspension of mesenchymal stem cells can be placed in a culture medium contained in a sterile conical-bottomed vessel (*e.g.*, polypropylene vessel). A plurality of cells (*e.g.*, about 200,000 to about 250,000

cells) can be placed in the vessel, and these cells can then be centrifuged to aggregate (*i.e.*, pellet) the cells. The resulting aggregates can be maintained in culture for several days to allow chondrogenesis to begin. After remaining in culture for several days, the cultured cells can be released from the aggregate environment by enzymatic digestion, and can then be used to seed large-scale tissue engineered constructs (*e.g.*, implants). Use of this method provides: markedly enhanced viability throughout the tissue engineered implants; chondrogenic differentiation of cells from mesenchymal stem cell preps that otherwise exhibited poor chondrogenic potential; and abundant chondroid extracellular matrix production.

Another aspect of the present invention relates to a pretreatment regimen to improve chondrogenesis by adult-bone marrow derived mesenchymal progenitor cells. In the method, bone marrow derived mesenchymal stem cells can be isolated from bone marrow biopsies. The cells can then be expanded in culture using a standardized set of culture conditions. Human recombinant fibroblast growth factor 2 (rhFGF-2) can be added to the human bone marrow derived mesenchymal stem cell culture at the first medium change (*e.g.*, on days 3 or 4) following isolation from the bone marrow biopsy, and throughout the entire monolayer culture expansion phase. The rhFGF-2 can be added to culture medium, for example, at about 1 to about 10 ng/ml final concentration, and the culture medium can be changed, for example, about two times per week. The cells can be passaged just prior to confluence. Use of this method provides marked enhancement (*e.g.*, about 2 to about 3 fold) of the proliferation rate of the cells in monolayer culture, markedly enhanced expression of markers of chondrogenesis by rhFGF-2 pretreated mesenchymal stem cells exposed to the appropriate stimuli, and rescue of mesenchymal stem cell preparations that displayed poor chondrogenic potential.

Yet another aspect of the present invention relates to a treatment regimen to improve chondrogenesis by adult-bone marrow derived mesenchymal progenitor cells. In the method, bone marrow derived mesenchymal stem cells can be isolated from bone marrow biopsies. The cells can then be expanded in culture using a standardized set of culture conditions. The expanded cells can then be seeded onto constructs (*e.g.*, biocompatible scaffolds). The assembled constructs can be grown in a continuous perfusion bioreactor with a chondrogenic medium that includes dexamethasone (*e.g.*, 10^{-7}

M). The dexamethasone concentration can be reduced in order to induce the internal synthesis of BMP-2, a chondrogenic growth factor.

BRIEF DESCRIPTION OF THE DRAWINGS

Further features of the present invention will become apparent to those skilled in the art to which the present invention relates from reading the following description of the invention with reference to the accompanying drawings in which:

FIG. 1 is a photograph illustrating a portion of a bioreactor system in accordance with an aspect of the present invention.

FIG. 2 is a photograph illustrating a portion of a bioreactor system with an internal mixing circuit in accordance with an aspect of the invention.

FIG. 3 is a photograph illustrating a portion of the internal mixing circuit of FIG. 2.

FIG. 4 is a photograph illustrating a portion of an optical pH measurement device in accordance with an aspect of the invention.

FIG. 5 is a photograph illustrating a portion of a fluid exchange system for a bioreactor system.

FIG. 6 is a photograph illustrating a portion of a bioreactor system in accordance with another aspect of the invention.

FIG. 7 is a photograph illustrating a portion of a bioreactor system in accordance with another aspect of the invention.

FIGS. 8A, 8B, and 8C are photographs illustrating bioreactor CFD modeling.

FIG. 9 is a photograph illustrating a portion of a bioreactor system and a hydrostatic loading device.

FIG. 10A is a photograph illustrating bioreactor equipped with hydrostatic loading module.

FIG. 10B is a photograph of a portion of the bioreactor of FIG. 10A.

FIG. 10C is a photograph illustrating the media control valve of the bioreactor of FIG. 10A.

FIG. 11 is a photograph illustrating a mesenchymal stem cell-based cartilage after 3 weeks of bioreactor culture.

FIG. 12 is a photograph illustrating a portion of the mesenchymal stem cell-based cartilage of FIG. 9.

FIG. 13 is a photograph illustrating the pH of the medium of bioreactor system in accordance with an aspect of the present invention.

FIG. 14 is a photograph illustrating the variations in color for culture mediums having varying pHs.

FIG. 15 is a photograph illustrating a portion of a multichannel fiber optic spectrophotometer for monitoring bioreactor medium pH in accordance with an aspect of the present invention.

FIG. 16 is a photograph illustrating a portion of the spectrophotometer of FIG. 13 attached to a bioreactor system.

FIG. 17 is a plot illustrating phenol red transmission spectra at pH 6.75 to 7.5.

FIG. 18 is a plot illustrating the correlation between test buffers and transmission at 559 nm.

FIG. 19 is a plot illustrating raw data taken from the spectrophotometer of FIG. 13.

FIG. 20 is a plot illustrating the raw data of FIG. 17 after it has been processed.

FIGS. 21A and 21B are plots illustrating forward scatter analysis of control hMSCs (top) and rhFGF-2 treated hMSCs (bottom).

FIG. 22 is a microphotograph illustrating aggregates made with control (left) and rhFGF-2 treated after 3 weeks in chondrogenic medium.

FIG. 23 is a photograph illustrating matched pairs of constructs from the same donor after 3 weeks in culture. (A): cells load immediately after monolayer culture. (B): cells loaded after 3 days in aggregate culture.

FIGS. 24A and 24B are photographs illustrating 3 week constructs. Top: cell pre-treated with FGF. Bottom: Dexamethasone withdrawn on day 6.

FIG. 25 is a photograph illustrating 3 week construct implementing combined FGF, aggregate pre-treatment, and dexamethasone withdrawal.

FIGS. 26A and 26B are photographs illustrating constructs labeled with 3, 10 and 70 kDa dextrans (L to R) at days 0 and 7. Fluorescent label shows as white.

FIG. 27 are plots illustrating the depth of penetration of each probe as function of MW.

FIG. 28 are photographs illustrating color contours representing fraction of atmospheric O_2.

FIG. 29 illustrates constructs assembled from cells preconditioned in aggregate culture for 3 (A) or 5 (B) days prior to assembly.

DETAILED DESCRIPTION

The present invention relates to apparatuses and methods for tissue engineering, and, particularly, to apparatuses and methods for generating tissue engineered constructs, such as tissue engineered cartilage implants for articular cartilage repair. One apparatus in accordance with the present invention relates to a bioreactor system that can provide multiple stimuli to cells simultaneously, and can be instrumented for diagnostic measurements. Referring to FIG. 1, the bioreactor system 10 can be modular and comprise an inner frame 12 that is sandwiched between two membranes 14 and 16 and two outer frames 18 and 20. The inner frame 12 can be of a basic biocompatible composition that can comprise suitable plastic, thermoplastic, synthetic, or natural materials, which can be fabricated into a framework structure, thereby achieving the required structural integrity for its intended purpose. It should be apparent to those skilled in the art that a wide latitude of choice can be exercised in selecting a material suitable for formation and/or fabrication of the inner frame 12.

The inner frame 12 can define a chamber 30, which forms the core of the bioreactor system 10. The chamber 30 can have a chamber volume that can contain a culture medium. The culture medium can be a liquid solution that can be used to provide sufficient nutrients (*e.g.*, vitamins, amino acids, essential nutrients, salts, and the like) and properties (*e.g.*, osmolarity, buffering) to maintain living cells (or living cells in a tissue) and support their growth. Commercially available tissue culture medium is known to those skilled in the art.

The dimensions of the inner frame 12 can depend on one or more factors including, but not limited to, the desired fluid capacity of the culture chamber formed therewith, and the dimensions of the culture chamber. In one aspect of the invention, the

inner frame can be substantially rectangular in shape. In another aspect of the invention, the inner frame can have a length, width, and height, which defines a culture chamber for culturing of cells in less incubator space than would be required for culturing cells at a comparable growth rate or to a comparable cell density using a conventional cell culture apparatus.

The inner frame 12 can include at least one inlet port 40 and outlet port 42that can serve as a passageway through which a substance can be introduced into and withdrawn or vented from the culture chamber30. It will be apparent to those skilled in the art that a substance that may be introduced into the culture chamber 30 of the bioreactor system 10 may include one or more of tissue culture medium alone, tissue culture medium with cells, physiological buffers, and/or a drug or cytokine or growth factor or enzyme (*e.g.*, solution of trypsin) or other biological agent to treat cells cultured therein. For example, the inlet and outlet ports 40 and 42 can be used to perfuse the culture chamber 30 with a culture medium (or media). Perfusion of the culture chamber 30 can be performed with a microprocessor controlled syringe pump. The culture medium perfused through the culture chamber 30 can be collected in waste containers and not recycled. This flow through approach avoids intermittent drastic changes in the extracellular environment associated with bulk medium exchanges and allows the establishment of a stable extracellular environment in the culture chamber.

Depending on factors such as the amount of substance introduced into the cell culture chamber 30, and the size of the port, it may sometimes be necessary to provide venting of the culture chamber 30. Venting is a process in which air or gas can be displaced from the culture chamber when the substance is introduced into the culture chamber 30r. Venting may be necessary to relieve pressure on the membranes of the cell culture chamber caused by the injection of air during the process of introducing the substance into the culture chamber.

The inner frame 12 can also include additional ports for instrumentation, sample collection, and media recirculation. For example, FIG. 2 illustrates that additional ports 50 can be provided for an internal mixing circuit, which circulates medium independent of the medium replenishment rate provide through the inlet and outlet ports. This allows

changes in hydrodynamics inside the chamber without increasing medium usage. It will be appreciated that other instrumentation can also be used.

The ports can be resealable by a suitable means known in the art such as a cap, a plug, or other suitable means. For example, the ports can be substantially filled and sealed with a material comprising gasket that is sufficiently pliable to be self-sealing, thereby allowing for penetration by a needle and resealing after needle withdrawal. Such material is known to those skilled in the art, and may include, but is not limited to one or more of rubber, silicone, silicone-rubber, or other elastomeric material suitable for the intended purpose. In another embodiment, the ports can be partially filled and sealed with gasket in forming resealable aperture that allows for penetration by a needle and resealing after needle withdrawal so as to prevent leakage from aperture out of culture chamber.

In accordance with an aspect of the invention, at least one of the membranes 14 and 16 can be a gas permeable membrane. The gas permeable membrane can comprise a biocompatible material, which is liquid impermeable, capable of allowing transfer of gases into and out of the cell culture chamber, and capable of excluding microbial contamination (*e.g.*, pore size is sufficiently small enough to exclude passage of microbes commonly encountered in contamination of cell cultures). The gas permeable membrane can be substantially rigid to permit hydrostatic loading of the culture cell without damage to the cultured cells. The gas permeable membrane can have an optical transparency and clarity for permitting observation of the cell culture (*e.g.*, of cultured cell characteristics such as growth and morphology of the cells as detectable by light microscopy). The thickness of the gas permeable membrane will depend on the desired resultant characteristics, which may include, but are not limited to, structural integrity, degree of gas permeability, and rate of transfer of gases.

The gas permeable membrane may be comprised of one or more membranes known in the art. Membranes typically comprise suitable polymers that may include polystyrene, polyethylene, polycarbonate, polyolefin, ethylene vinyl acetate, polypropylene, polysulfone, polytetrafluoroethylene, or a silicone copolymer. The choice of the composition of the gas permeable membrane will depend on the type of cell to be cultured (*e.g.*, cells which grow attached (anchorage-dependent), cells which grow in

suspension (anchorage-independent), cells that may grow as attached or in suspension), degree of gas permeability, rate of transfer of gases, and optical transparency and clarity. For example, in accordance with one aspect of the invention, the membrane can be a fluoro-ethylene-propylene (FEP) membrane (*e.g.*, FEP (Teflon®) membranes). The gas permeability of FEP is appropriate for biologically relevant gases at nominally 1.6×10^3 and 25.9×10^3 cm^3/m^2-day-atm for O_2 and CO_2, respectively across a 0.026 mm film at 25°C.

It may be desirable to have both membranes 14 and 16 comprise gas permeable membranes in order to allow relative uniform gas exchange and equilibrium in the culture medium. Additionally, both gas permeable membranes may simultaneously serve as surfaces for attachment of anchorage-dependent cells cultured in the bioreactor system. Both membranes can be of a sufficient optical transparency and clarity so as to permit observation during culture, such as of the color of the tissue culture medium, and of cellular characteristics (*e.g.*, growth and morphology of cells such as by microscopy). Membranes can be of a sufficient optical transparency and clarity so as to observe during culture, changes in the color of the medium's pH indicator. Additionally, both membranes can be of a sufficient optical transparency and clarity so that when cell culture apparatus is analyzed, for example with a microscope, the cultured cells therein may be visually analyzed for cell shape, cell number, and additional cell characteristics that typically can be observed by light microscopy.

Optionally, at least a portion of one of the membranes or both of the membranes can be substantially gas impermeable, that is incapable of exchanging gas sufficiently to support the growth of cultured cells in the absence of another source for gas exchange. For example, it may be desirable for both membranes to be gas-impermeable for diagnostic applications. In this application, the contents of the bioreactor can isolated so as to measure oxygen consumption.

The gas impermeable membrane can comprise a biocompatible material which is liquid impermeable, which is capable of excluding microbial contamination (*e.g.*, pore size is sufficiently small enough to exclude passage of microbes commonly encountered in contamination of cell cultures), and which is optically transparent and clear for permitting observation during the cell culture process. Thickness and/or choice of

composition of the impermeable membrane will depend on the desired resultant. The impermeable membrane may be comprised of one or more membranes known in the art. The impermeable membrane may be treated, on a side of the membrane, which may serve as a surface for attachment of anchorage-dependent cells in culture.

The membranes 14 and 16 can be sealed to the surfaces of the inner frame 12 in a leak-proof sealing using means that may include mechanical means, chemical means, or other suitable means. For example, a mechanical means, such as a clamp or screw can be used to secure the membranes to the inner frame 12. Gaskets (*e.g.*, silicone rubber gaskets) can be provided between the inner frame 12 and membranes 14 and 16 to form a leak-proof seal between the inner frame 12 and the membranes 14 and 16. In another example, chemical means, such as the use of an adhesive agent (also encompassing a bonding agent) may be used to secure the membranes 14 and 16 to the frame 12 in forming a leak-proof seal. The adhesive agent may be in the form of a double-faced adhesive tape, a polymeric adhesive, a pressure-sensitive acrylic adhesive, hot-melt adhesive, rubber cement, or any other form of adhesive or bonding agent useful for the purposes attendant to the present invention. Other suitable means may include one or more of heat bonding, sonic welding, pressure fit sealing in forming a leak-proof seal, and a molding process in which the membranes become an integral part of the frame. For example, in using an adhesive agent, the adhesive agent can be applied between the frame and the portion of each membrane which can extended over the frame such that the portion of the membranes that extend over the frame contacts the adhesive agent on the frame surface, and pressure may be applied to cause a force along the horizontal axis of that portion of the membranes being secured to the frame in a manner which results in a leak-proof sealing between the membranes secured to the frame in the formation of a culture chamber.

The inner frame 12 and the membranes 14 and 16 can be sandwiched between the outer frames18 and 20. The outer frames 18 and 20 can secure the membranes 14 and 16 to the inner frame 12 by mechanical means, such as screws or clamps, or other suitable means. The outer frames 18 and 20 can have substantially rectangular shapes that can accommodate the aligning, contacting and securing thereto (in a leak-proof sealing) the membranes to the inner frame 12 in assembling bioreactor system 10. The outer frames

18 and 20 can include openings 60 and 62 that can allow for continuous or cyclic hydrostatic pressure in the Mpa range to the to the culture chamber 30. The outer frames can be formed from a substantially rigid material, such as a metal (*e.g.*, aluminum). It will be appreciated by one skilled in the art that other materials can be used to form the outer frames.

The bioreactor system 10 can further include monitoring systems and mechanical stimulation modules that can be used to monitor and control the culture media, the culture chamber environment, and the growth of the cells or tissue in the culture chamber. Environmental parameters that can be monitored and controlled include pH, temperature, solute concentration, and oxygenation, CO_2 concentration, and glucose concentration. It will be appreciated by one skilled in the art that other selected products or conditions can also be monitored or affected. In one aspect of the invention the monitoring system can be set to clamp selected environmental parameters at predetermined values, while allowing others to float as outcome variables, and can be set to flag specific conditions as requiring intervention.

FIG. 4 illustrates an example of a monitoring system in accordance with an aspect of the present invention. The monitoring system can include a multi-channel fiber-optic spectrophotometer. The spectrophotometer can measure light transmission and absorbance of at least one molecular species in at least one sample. For example, the spectrophotometer can be used to continuously measure the pH of the cell culture medium at an inlet and an outlet of a culture chamber. In this example, the spectrophotometer can capture the phenol red absorption spectra at 559 nm. The spectrophotometer can include a monochromator that can be modified to position polished ends of optical fibers (*e.g.*, 500 μm) in an output slit plane. The wavelength can be adjusted using a stepper motor (40 steps/nm). Flow-through cuvettes can be positioned at the inlet and outlet of each culture chamber. Cuvette holders can align the collimated output from each fiber, the cuvette, and a detector-amplifier hybrid. Data collection and control can be synchronized using custom software. The correlation (r^2 = 0.998) between pH and transmission at 559 nm in the physiologically relevant range permits calibration of the device in pH units. pH differences of 0.05 units were readily

resolved. Better resolution can be expected with sampling at more wavelengths. Repeat measurements of the same samples correlated with an r^2 of better than 0.99999.

The bioreactor system can be used to culture cells, whether the cells are individual cells (cells which are grown independent of forming a structure such as a tissue; an illustrative example being a cell line), or cells forming a tissue (typically, a mesh or network of cells, with their intercellular substance in forming a structured or organized tissue), or a combination thereof. It will be apparent to one skilled in the art that individual cells which can be cultured in the bioreactor system comprise one or more cell types including, but not limited to, animal cells, insect cells, mammalian cells, human cells, transgenic cells, genetically engineered cells, transformed cells, cell lines, anchorage-dependent cells, and anchorage-independent cells. It will be also apparent to one skilled in the art, that tissue formed by cells in culture can also be cultured in the bioreactor system according to the present invention. It will further be apparent to one skilled in the art that the bioreactor system of the present invention is generally not limited to a specific type of cell to be cultured, nor the tissue culture medium capable of sustaining cell growth as long as the medium provides sufficient nutrients and properties (*e.g.*, osmotic pressure) to maintain and support cell growth.

In accordance with another aspect of the invention, the cells cultured in the bioreactor system can include bone marrow-derived human mesenchymal stem cells. These cells can be vacuum-seeded on porous scaffolds, may or may not be biodegradable depending on the intended application. Examples of scaffolds can include hyaluronan-based scaffolds (Hyaff-11, Fidia advanced Biopolymars, IT), gelfoam, sponges, such as collagen-based sponges, cross-linked collagen-GAG copolymers sponges or PLLA or PLGA-based scaffolds, porous calcium phosphate ceramics, or titanium meshes. It will be appreciated by one skilled in the art that other scaffolds can also be used.

The seeded scaffolds (*i.e.*, tissue engineered constructs) can be introduced into the culture chamber of the bioreactor system. While in the culture chamber of the bioreactor system, the cells can be continuously perfused with a tissue culture medium. The bioreactor system can provide conditions sufficient for cell growth as known in the art (*e.g.*, a closed environment with controlled atmospheric conditions including suitable temperature and CO_2 concentration).

It is noted here that the step of introducing the cells and tissue culture medium into the cell culture chamber can be performed in a sterile environment, or non-sterile environment provided that aseptic technique is used. This is because the bioreactor system itself (when sterilized using conventional means known in the art) can provide a sterile, hermetic environment.

The cells can be manipulated before and after assembly into the constructs to reduce the consumption of externally supplied substrates, and thus enhance viability and chondrogenesis. For example in one aspect of the invention, a suspension of mesenchymal stem cells can be placed in a culture medium with chondrogenic supplements contained in a sterile conical-bottomed vessel (*e.g.*, polypropylene vessel). A plurality of cells (*e.g.*, about 200,000 to about 250,000 cells) can be placed in each vessel, and these cells are then centrifuged to aggregate the cells. The resulting aggregate can then be maintained in culture for several days to allow chondrogenesis to begin. After remaining in culture for several days, the cells can be released from the pellet environment by enzymatic digestion, and are then used to seed large-scale tissue engineered implants. Use of this method provides markedly enhanced viability throughout the construct, chondrogenic differentiation of cells from mesenchymal stem cell preps that otherwise exhibited poor chondrogenic potential, and abundant chondroid extracellular matrix production.

In another aspect of the invention, a pretreatment regimen can be used to improve chondrogenesis by adult-bone marrow derived mesenchymal progenitor cells. In the method, bone marrow derived mesenchymal stem cells are isolated from bone marrow biopsies. The cells can then be expanded in culture using a standardized set of culture conditions. rhFGF-2 can be added to the human bone marrow derived mesenchymal stem cell culture at the first medium change (*e.g.*, on days 3 or 4) following isolation from the bone marrow biopsy, and throughout the entire monolayer culture expansion phase. The rhFGF-2 can be added to culture medium, for example, at about 1 to about 10 ng/ml final concentration, and the culture medium can be changed, for example, about two times per week. The cells can be passaged just prior to confluence. Use of this method provides marked enhancement (*e.g.*, about 2 to about 3 fold) of the proliferation rate of the cells in monolayer culture, markedly enhanced expression of markers of

chondrogenesis by rhFGF-2 pretreated mesenchymal stem cells exposed to appropriate stimuli, and rescue of mesenchymal stem cell preparations that otherwise displayed poor chondrogenic potential.

In a further aspect of the invention, bone marrow derived mesenchymal stem cells can be isolated from bone marrow biopsies. The cells can then be expanded in culture using a standardized set of culture conditions. The expanded cells can then be seeded onto constructs (*e.g.*, biocompatible scaffolds). The assembled constructs can be grown in a continuous perfusion bioreactor with a chondrogenic medium that included dexamethasone (*e.g.*, 10^{-7} M). The dexamethasone concentration can be reduced in order to induce the internal synthesis of BMP-2, a chondrogenic growth factor.

EXAMPLES

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

Example 1

Modular Bioreactor System

Introduction:

In vivo, the majority of mass transport takes place via the microvascular network of blood vessels as small as 5μm in diameter, which continually supply nutrients and eliminate waste products from tissues. Almost every metabolically active cell in the human body is within a distance of 50μm from a blood capillary. Some tissues, in particular cartilage, lack an intrinsic vasculature; the cells in these tissues are adapted to survive off diffusion from the adjacent vascularized tissues.

We are attempting to generate tissue engineered cartilage constructs based on mesenchymal stem cells (MSCs), which are then driven to differentiate to cartilage. Unlike cartilage cells, MSCs, which are derived from well-vascularized bone marrow cells have high metabolic requirements. To support and maintain the cells and their functions in these constructs during differentiation, measures must be taken to provide nutrients, growth factors, an gas exchange, and to remove waste products.

As a first step towards improving the transport of nutrients, growth factors, and waste products to and from the cells in our constructs, we have developed a simple, but modular continuous perfusion bioreactor system.

Furthermore, as synergistic effects of, for example, perfusion, gas exchange and mechanical stimulation have been noted, we have developed our system to be able to provide multiple stimuli to the cells simultaneously, as well as be instrumented for diagnostic measurements.

The bioreactor chamber forms the core of a modular system to which monitoring instrumentation and mechanical stimulation modules can be added. When this system is fully developed, environmental parameters including pH, temperature, solute concentrations, and oxygenation will be continuously monitored, and mechanical stimulation will be provided, all following a pre-defined profile under automated computer control without exposing the contents of the bioreactor. The monitoring system can be set up to clamp selected environmental parameters at specific predetermined values, while allowing others to float as outcome variables, and can be set to flag specific conditions as requiring intervention. These features are useful if this apparatus is further developed for use in a clinical setting, where maintaining a sterile environment is critical.

Finally, with a view towards adapting this technology for clinical use, a constant goal in developing our bioreactor has been to create a self-contained system, which, after being assembled in a sterile environment, does not need to be opened until the end of the experimental run.

Materials and Methods

Gas Exchange:

To enable gas exchange across an otherwise closed system, we use fluoroethylene-propylene (FEP) membranes. The gas permeability of FEP is quite good for biologically relevant gases, at nominally 1.6×10^3 and 25.9×10^3 cm^3/m^2-day-stm for O_2 and CO_2, respectively across a 0.026 mm film at 25°C.

Fluid Exchange:

FIG. 5 illustrates that the chamber can be perfused with cell culture medium using a microprocessor controlled syringe pump (Harvard instruments, Holliston, MA). The medium is collected in waste containers and is not recycled. The flow-through approach

avoids the intermittent drastic changes in the extracellular environment associated with bulk medium exchanges, and allows the establishment of a stable extracellular environment.

Other Environmental Controls:

To maintain the internal temperature, the system is housed in an incubator at 37°C with a 7.5% $C0_2$ atmosphere. Mixing of the internal environment is improved by placing the bioreactors on a rocking platform and rocking them through at 30° arc at 0.5 Hz.

Bioreactor Mk 2:

FIG. 6 illustrates an example of a portion of a bioreactor system. The bioreactor system includes an FEP® fluorocarbon based on a commercial design by American Fluoroseal Corporation (AFC, Gaithersburg, MD). In collaboration with AFC, their bag design was modified into a flow-through version with Luer ports at either end, scaffolds shipped to AFC were inserted into the bags during manufacture, this made loading the cells onto the metrics through the bioreactor ports cumbersome and inefficient.

Bioreactor Mk 3:

FIG. 7 illustrates a portion of a bioreactor system of FIG. 6 that was modified to include a large sideport, which allowed us to seed the matrices with cells outside of the bioreactor, and develop better leading methods.

Bioreactor MK 5:

Referring to FIG. 1, this version of a portion of the bioreactor system retains the FEP membrane for gas exchange, but these are mounted in a rigid aluminum/Delrin frame and sealed with silicone rubber gaskets. Advantages are constant geometry, and better protection of the contents. The center frame is replaceable, and in addition to the media inlet and outlet ports, additional ports for instrumentation or sample collection can be fitted. Chamber volume is controlled by the thickness of the central Delrin frame.

Bioreactor MK 6:

Referring to FIGS. 2 and 3, an internal mixing circuit circulates the medium independent of the medium replenishment rate. This allows changes in the hydrodynamics inside the chamber without increasing medium usage.

Instrumentation:

Referring to FIG. 4, a bioreactor chamber can be fitted with flow-through spectrophotometric cuvettes on the inlet and outlet ports. In this case, the system is used as a testbed for a novel solid-state iridium oxide based pH electrode/reference electrode pair seen protruding though the supplementary Luer fitting on the right. Optical pH measurement will be used to validate the sensor data. Other applications include monitoring medium consumption rate. These data can be used as part of a feed-back loop to dynamically control the medium perfusion rates.

CFD modeling:

FIG. 8 shows CFD modeling of our bioreactor system. The computational domain was discretized (A) using a mesh consisting of 5500 tetrahedral elements (the geometry being symmetrical, only half of the domain was used in computations). Navier-Stokes, continuity and oxygen concentration equations in regions R_1 and R_2, and the oxygen concentration equation without convective terms was solved in region R_3 were solved simultaneously using a computational fluid dynamics solver (CFX, ANSYS Inc, Canonsburg, PA). The incoming fluid and the fluid at the FEP membrane interface were considered to be saturated with oxygen. Oxygen is consumed in the disc by Michaelis-Menten kinetics with V_{max} equivalent to that of chondrocyte monolayers. A K_m equivalent to 20% of water saturation value of oxygen in air was assumed.

The preliminary results show that although the bulk of the medium (region R_1) was saturated with 21% oxygen, in a bioreactor without additional internal mixing, steep oxygen gradients exist in region R_2 (B as stream lines, and C as concentration profile). The mass transfer resistance offered by this boundary layer region outside the construct can be greatly decreased by improving hydrodynamic conditions in the bioreactor. One such improvement involves the use of internal mixing to disrupt the resistance in R_2. Additional modeling efforts are currently underway which will account for internal mixing. The computational fluid modeling will also be used to predict the shear stress distribution at the surface and inside the construct. This will help us avoid shear stress conditions unsuitable for tissue growth and optimize the hydrodynamic conditions inside the tissue engineered construct.

Hydrostatic loading:

FIG. 9 illustrates an external module allows the application of continuous or cyclic hydrostatic pressure in the MPa range to the contents of the bioreactor chamber. Computer controlled pumps pressurize the outer chamber, hydrostatic pressure is transmitted through the FEP membrane to the contents of the bioreactor.

The hydrostatic loading system will incorporate environmental controls systems which will make the device stand alone, i.e. independent of a cell culture incubator. These environmental controls include water-to-media based heat and gas exchange and can be used independently of the application of hydrostatic pressure. For example, pressure can be applied, but does not have to be. This will allow cells from other patients to be kept physically separated.

Hydrostatic loading chamber development:

We have completed development of a working, fully automated prototype (FIG. 10) for the hydrostatic loading chamber. Salient developments are a new, improved valve design for the media inlet and outlet, with improved sealing and better duty cycle capability, as well as completion and successful testing of the control software for the hydrostatic loading and the environmental controls (temperature, CO_2). Arbitrary loading/perfusion regimens can now be implemented using the software. The environmental controls will allow the bioreactor to be operated independently of a cell culture incubator. Together these permit long-term pressure cycling without operator intervention, and, for future clinical trials/applications permit the physical segregation of implants generated from individual donor material.

FIG. 10A shows the bioreactor equipped with a hydrostatic loading module. FIG. 10B shows a (A) bioreactor core. Media control valve (B) shuts off media feed line (C) during pressurization. Hydrostatic pressure provided by heated, CO_2 equilibrated, water circulating through lines (D), and is monitored by pressure sensor (E). FIG. 10C shows media flow control valve detail – upper row: electromagnetic coil and housing, below; valve seat, valve and spring assembly.

Results Summary

FIG. 11 illustrates a successful, 7 x 2 mm MSC-based cartilage implant after 3 weeks of bioreactor culture. Toluidine blue staining. FIG. 12 illustrates higher

magnification immunofluorescence for type of collagen, a marker of chondrogenesis (green). Red staining is a fluorescent dextran tag, blue is DAPI nuclear counterstain (bottom).

Example 2

A Multichannel Fiberoptic Spectrophotometer for Monitoring Bioreactor Medium pH

Introduction:

Miniaturized bioreactor systems are becoming an increasingly useful tool in tissue engineering applications. Our laboratory is currently exploring their use for the generation of large cartilage implants for the repair of cartilage defects. In these studies, we assemble mesenchymal precursor cells at very high densities (up to 100 x 10^6 cells/ml) and carrier matrices to form composite grafts of up to 2x2 cm. The precursor cells are then induced to differentiate along the chondrogenic pathway in a bioreactor system.

A major technical problem associated with the *in vitro* maintenance of large constructs, in which tens of millions of cells are bathed in a few ml of culture medium, is the maintenance of an appropriate supply of fresh medium. The replacement rate must be adequate to satisfy the metabolic needs of the cells, to supply needed growth factors, and to wash out waste products (FIG. 13). Offsetting this requirement is the goal of not washing out potentially useful autocrine or paracrine factors secreted by the cells.

A useful marker for the extent to which the cell culture medium has been depleted by the cells is the pH of the medium. Over time, the medium pH drops due to cell metabolism and the release of lactate and other waste products (FIG. 14). In our system, the medium pH drops from about pH 7.3-7.4 at the inlet to pH 6.5 at the outlet of the bioreactor.

The purpose of this study was to develop a non-contact optical system to continuously measure the pH of the cell culture medium at the inlet and outlet of multiple bioreactor chambers. Most common cell culture media contain a pH indicator dye, which shifts color from red to yellow in the range of ph usually encountered in cell culture environments. This color shift is due to a strong, pH sensitive light absorption peak at 559 nm of the indicator dye phenol red, and this is exploited in the current approach.

Materials & Methods:

The following components were developed for this project.

Multi-channel fiber optic spectrophotometer:

FIG. 15 illustrates an example of a portion of a spectrophotometer in accordance with an aspect of the invention. As a tunable source of monochromatic light, we used the monochromator from a Beckman model DU-8 spectrophotometer, illuminated by a 12V50W tungsten lamp. The monochromator was modified to position the polished ends of 500 μm optical fibers in a 5 x 13 array in the output slit plane. The monochromator output wavelength can be adjusted dynamically using a computer-controlled stepper motor (0.025 nm/step).

Flow-through cuvettes with a 1 cm optical path length were designed to be positioned at the inlet and outlet of each bioreactor chamber. They can be cleaned and sterilized independently of the cuvette holders.

Cuvette holder:

FIG. 16 illustrates the cuvettes are placed in an opaque black Delrin cuvette holder. A modified SMA connector houses and aligns a single fiber, a 1.5mm sapphire ball lens is used to collimate the light output. A blue-enhanced detector-amplifier hybrid (PDB-716-100, Photonic Detectors Inc.) collects light transmitted through the sample. The output voltage is read on a HP 3421A voltmeter. Data collection and stepper motor control are synchronized using custom PC based software.

Test samples:

To ensure accurate test pH values, samples were 200 mM phosphate buffer adjusted to pH 6.75 to 7.50 in 0.05 pH increments. All contained phenol red at 15 mg/l – identical to most commercial medium formulations. pH of the test samples was verified using an Accumet pH meter with a glass Ag/AgCl electrode. In the wavelength range (400-700 nm) tested, these samples had absorption spectra which were essentially indistinguishable from that of normal culture medium. Test samples were drawn into the cuvette and then scanned from 400-700nm. Cuvettes were flushed with dH_20 between readings.

Results Summary:

FIG. 17 is a plot illustrating phenol red absorption spectra were successfully captured using the multi-channel fiber optic spectrophotometer. The predicted peak absorption at 559 nm was readily detectable. A scan of deionized water was used as a blank to normalize all the spectra, to account for spectral non-linearities in the optical components of the system. pH differences of 0.05 units were readily resolved using only the transmission at 559nm. Better resolution can be expected with sampling at more wavelengths.

FIG. 18 is a plot illustrating the strong, linear, correlation ($r^2 = 0.998$) between pH and transmission at 559 nm in the physiologically relevant range permits easy calibration of the device in pH units. Repeat measurements of the same samples correlated with an r^2 of better than 0.99999.

Three channels of data are currently collected. FIGS. 19 and 20 illustrate, respectively, raw and processed data taken from the instrument. In addition to the inlet and outlet of the bioreactor, a water-filled cuvette serves as a blank to normalize the readings. Although only 3 channels are currently used, the system allows expansion up to 65.

Our immediate goal in designing this system was to use the medium pH as a marker for the extent to which the culture medium in the bioreactor had been metabolized. Our fiber optic spectrophotometer appears suitable for this task. Major advantages of this device include that the medium pH can be continuously sampled in a non-contact manner, without breaching an otherwise closed bioreactor system, and that a large number of channels can be sampled simultaneously.

Output from this device can be used to measure the rate at which cells deplete their culture medium at a given flow rate. In addition, this information can be used to continuously modulate the media flow rates in the bioreactor using medium pH as the control variable, to maintain the pH within a desired range. Alternatively, the output could be used to modulate the pH by controlling the PCO_2 in the incubator.

Example 3

A Pre-treatment Regimen To Improve Chondrogenesis By Adult Bone-marrow Derived Mesenchymal Progenitor Cells

Background:

Successful tissue engineering of articular cartilage has the potential to revolutionize the therapy of degenerative joint disease. Adult human mesenchymal stem cells (hMSCs) are attractive candidates for this role due to their documented osteogenic and chondrogenic potential, and ease of harvest and mitotic expansion. For this purpose, mesenchymal stem cells have to be harvested from the donor, and expanded in culture, sometimes considerably, in order to obtain sufficient numbers of cells to seed biodegradable scaffolds at high densities. To obviate immunological complications, the use of autologous cells is preferred. The cells are isolated from a bone marrow biopsy and must be expanded in culture. This adds a lag time of several weeks between the initial harvest of the cells, and the implantation of the completed construct into the cartilage lesion. Any approach which has the potential to shorten this interval, or to improve the creation of the construct, would therefore be desirable.

Growth factors have the capacity to modulate or modify the phenotype of cells exposed to them. Desirable modulations of the phenotype which can improve the tissue engineering process include: enhancement of mitogenic potential (which results in a significantly shortened time in culture), maintenance of a pluripotential, or enhancement of the chondrogenic potential

In preliminary tests of this method, as outlined below, we have achieved at least two of these three desired modulations.

Methods

Bone marrow derived mesenchymal stem cells were isolated from marrow biopsies following standard published procedures. The cells were then expanded in culture using a standardized set of culture conditions. The cell preparations were subjected to a density gradient and plated in control medium (DMEM-LG+10% FBS). Our modification to the method consists in the addition of recombinant human FGF-2 (rhFGF-2) to the human bone-marrow derived mesenchymal stem cell culture at the first medium change on days 3 or 4 following isolation from the bone marrow biopsy, and

throughout the entire monolayer culture expansion phase. The rhFGF-2 was added to the culture medium at 1-10 n ng/ml final concentration, and the culture medium is changed 2 times per week. Cells are passaged just prior to confluence.

At the first medium change, (day 4) the human bone-marrow derived mesenchymal cell cultures either received control medium, or medium supplemented with rhFGF-2 at 10 ng/ml. Cell numbers and size were evaluated by flow cytometry at the end of primary and first passage; at the end of first passage each subpopulation was introduced into aggregate culture to induce chondrogenic differentiation. No FGF-2 was present in the aggregate culture medium. RNA was extracted at the end of first passage, and gene expression profiles for each group were generated using the Affymetrix HG-U133A chip. Pairwise comparisons using the Affymetrix MAS algorithm and conservative acceptance criteria were used to identify differential gene expression.

Results

Mitotic effects:

Human MSCs expanded in the presence of rhFGF-2 exhibit shorter population doubling times and are reproducibly smaller than those maintained in control conditions (FIGs. 21A and 21B).

Chondrogenic potential:

rhFGF-2 treated cells produce aggregates that differentiate more rapidly and are significantly larger and more homogeneous (FIG. 22) than those generated with control cells.

Microarray analysis yielded a database of more than 700 genes differentially expressed (2-fold or greater change) in the two groups.

Discussion & conclusions

Exposure of hMSCs to rhFGF-2 during mitotic expansion increases cell yield and shortens time in culture. In preliminary experiments, rhFGF-2 treated cells undergo 3 more population doublings than Control cells by the end of first passage, and up to 10 more by the end of fifth passage. The effects of transient rhFGF-2 treatment of the monolayer cells persist when chondrogenesis is induced. Morphometric and biochemical analyses suggest that the increased size of the aggregates is due to increased matrix production by the rhFGF-2 treated hMSCs, and not due to higher number of cells in the

aggregates. The mechanism behind this increased synthesis of extracellular matrix has not yet been determined, but the microarray data have provided several candidate genes involved in cell communication and signal transduction (Table). Several of these genes have been reported to participate in the regulation of chondrogenesis and may, therefore, be involved in this enhancement of chondrogenic differentiation induced by exposure to rhFGF-2. Both the mitotic and chondrogenic enhancements could be substantial advantages when using these cells in cartilage repair.

Table

List of signal transduction related genes differentially expressed in FGF-treated cells

Down-regulated	
Fold-change	**Symbol**
-19.28	CLIC3
-9.22	WISP1
-5.09	TNFRSF11B
-4.57	MME
-4.06	PDK1
-3.83	VLDLR
-3.31	PENK
-3.25	INHBA
-2.92	IGFBP3
-2.89	LIM
-2.83	FN1
-2.79	EDN1
-2.72	GADD45B
-2.64	PDGFA
-2.59	CELSR1
-2.58	JAG1
-2.54	RGS4
-2.52	FZD7
-2.47	IL6
-2.43	LEPR
-2.29	TGFBR1
-2.29	FLNB
-2.22	STC2
-2.20	PTPRF
-2.17	PTPLA
-2.10	CDH2
-2.08	FGF2
-2.05	STAT4
-2.05	LYN
-2.02	SIAH2
-2.01	TGFB2
-2.00	TRAF5

Up-regulated	
Fold-change	**Symbol**
17.21	PTPN22
10.38	CXCL6
9.99	DUSP4
8.75	HTR2B
7.44	HLA-DRB3
7.35	SRFP1
6.55	BMP2
6.29	TRHDE
5.55	DUSP6
5.01	EDNRA
4.30	HGF
4.30	HLA-DRB4
4.23	ARHGEF3
4.18	F2RL1
4.14	PTGER2
3.35	GRB14
3.31	BDKRB2
3.29	RDGBB
3.09	HMGA2
3.07	GEM
3.05	NDP
2.83	SPRY1
2.60	IL7
2.56	RGS2
2.56	PPRGC1
2.44	PPKAR2B
2.43	CCL2
2.31	PBEF
2.22	SPRY2
2.18	TGFBR3
2.16	NET1
2.15	TNFAIP6
2.07	TGFBR2
2.05	LIFR
2.03	PTGFR
2.02	MAP4K4

Example 4

A Method to Improve Chondrogenesis by Adult Mesenchymal Stem Cells

Background:

Successful tissue engineering of articular cartilage has the potential to revolutionize the therapy of degenerative joint diseases. This is particularly true of cartilage tissue engineering based on bone marrow-derived mesenchymal stem cells. This approach has the potential to allow the creation of very large amounts of new cartilage tissue for implantation without the need for harvesting healthy articular cartilage from a non-affected area.

A common implementation of cartilage tissue engineering, which we use as well, is to seed a biodegradable carrier scaffold with cells. The carrier scaffold provides the initial structural properties of the construct, and is then gradually replaced by the cellular component and its products. To support and maintain the cells and their functions during this process, nutrients must be provided to the cells, and waste products must be cleared. tissue engineered constructs in general, and tissue engineered cartilage in particular, do not have a built in vasculature. Therefore, the transport of these molecules to and from the cells inside the engineered tissue mass must occur by diffusion. Chondrocytes in their natural environment (2-3 mm thick cartilage) survive for decades and product cartilaginous matrix. This suggests that these terminally differentiated cells are adapted to meet their basal metabolic requirements through diffusion. There are at least three sources of problems, which complicate the routine generation of mesenchymal stem cell-based tissue engineered cartilage implants. This aspect of the invention targets all three of these.

At early time points, the tissue engineered constructs are populated by mesenchymal stem cells which are not (yet) adapted to survival under these conditions. Limiting factors are the dimensions of the construct itself, and the metabolic requirements of the cells contained therein. Large constructs therefore face mass transfer issues that lead to limited function and viability. In constructs seeded with mesenchymal stem cells and treated with chondrogenic medium in a bioreactor environment, chondrogenesis begins at the construct surface and proceeds in a centripetal fashion resulting in the formation of a shell of differentiated material surrounding a viable, but

undifferentiated core. This is not the desired outcome, which would be a uniform construct.

In addition, mesenchymal stem cells are a non-homogeneous cell population with differing chondrogenic potential. In micromass and aggregate (*i.e.*, pellet) cultures, cells, which do not take part in chondrogenesis, are shed from the construct. The size and shape of tissue engineered constructs, and the assembly methods used do not allow for the cells to self-sort according to chondrogenic potential.

Finally, the ability of mesenchymal stem cells to differentiate into cartilaginous tissue in tissue engineered constructs differs markedly from preparation to preparation. In contrast, almost all preparations undergo differentiation in micromass or aggregate/pellet culture. This is likely due to the combination of the first two issues.

Method:

In several model systems of chondrogenesis, *e.g.*, micromass and aggregate/pellet culture, the diffusion distances are reduced to the point where mass transport issues become less dominant. In addition, it allows for a sorting process to take place, in which only certain subpopulations of the mesenchymal stem cell preparation take part in the cartilage formation. Under these conditions, the bulk of the mesenchymal stem cells, which take part in the aggregate/pellet formation differentiate to become chondrocytes. However, this results in constructs are so small as to be useless from a therapeutic standpoint.

Our new method takes advantage of the aggregate/pellet culture approach, where mass transport is not limiting, to drive the cells down the chondrogenic lineage. In addition, cells which do not take part in chondrogenesis, are selectively excluded during this period.

Briefly, the aggregate/pellet culture approach is as follows: a suspension of mesenchymal stem cells are placed in sterile conical-bottomed polypropylene vessels in cell culture medium with chondrogenic supplements. About 200,000 to 250,000 cells are placed in each vessel, and these are then centrifuged to aggregate/pellet the cells. The resulting aggregate/pellets are then maintained in culture for several days to allow chondrogenesis to begin.

The innovation we are introducing is that, at this point, the cells are released from the aggregate/pellet environment by enzymatic digestion, and are then used to seed large-scale tissue engineering implants.

Results:

Tests of this method are highly encouraging, yielding: markedly enhanced viability throughout the construct, chondrogenic differentiation of cells from mesenchymal stem cell preps that otherwise exhibited poor chondrogenic potential abundance chondroid extracellular matrix production.

Example 5

Modulation of Growth and Differentiation Conditions Enhances Chondrogenesis In Large Tissue Engineered Constructs.

Introduction:

Tissue engineered implants based on bone marrow derived mesenchymal stem cells (MSCs) are a promising approach to articular cartilage repair. Substrate (e.g., nutrients, waste products) mass transport considerations are critical for any tissue-engineered construct, but our constructs initially contain particularly large numbers of metabolically very active cells. As the constructs mature, abundant, diffusion limiting extracellular matrix is produced. Some substrates that are absolutely required for the induction of chondrogenesis in MSCs are of high MW (e.g., TGF-β1 – 25kDa) and have small diffusivity (10^{-7} cm^2/sec). Constructs frequently exhibit a peripheral cartilaginous shell, with limited cell function at the center, as substrate availability is limited by both diffusion and cellular consumption. These two limiting factors are targets for interventions designed to improve chondrogenesis. In this study, we hypothesized that manipulating the cells before and after assembly into the constructs could reduce the consumption of externally supplied substrates, and thus enhance viability and chondrogenesis.

Materials and Methods:

Constructs:

Control MSCs were isolated, culture expanded for 2 passages in DMEM + 10% FBS, and then vacuum-seeded onto 7.3 mm on porous scaffolds. In this case the outcome

of our manipulations was tested on a Fidia, Hyaff-11® material (Fidia, Abano Terme, Italy), as the carrier scaffoled at a density of 10^8 cells/ml, as described previously. Other scaffolds can be used as well. The assembled constructs were grown for 3 weeks in a continuous perfusion bioreactor. Baseline chondrogenic medium was DMEM-HG with 1% ITS+Premix™, 100 μM ascorbate-2-phosphate, 10^{-7} M dexamethasone and 10 ng/ml TGF-β1. Three experimental treatments, described below, were used, individually or in combination.

Preconditioning in aggregate culture:

In this approach, the expanded cells were pre-conditioned in aggregate culture at $2.5x10^5$ cells per aggregates by enzymatic digestion, and then assembled into the large constructs as described above. Our working hypothesis is that the initial stages of differentiation can be bridged in the aggregate environment (1-2mm diameter spheres) where substrate diffusion is less limiting.

Growth factor pretreatment:

We have tested several growth factor-based modifications to the growth medium. For this study, the medium was supplemented with 10ng/ml FGF-2 beginning in primary culture. The mechanism underlying the FGF response remains unclear.

Dynamic modification of the chondrogenic medium:

We have modified the composition of the baseline chondrogenic medium by adding or withdrawing components at defined points during the culture process. In this study, we have tapered the dexamethasone concentration to 0 beginning at day 6. The working hypothesis is that dexamethasone withdrawal induces the internal synthesis of BMP-2, a chondrogenic growth factor.

Outcome assessment:

At the end of 3 weeks in bioreactor culture, the implants were harvested and processed for standard histology and collagen immunohistochemistry. A subset of the samples was tested mechanically to determine bulk material properties of the constructs.

Results:

Individually, each of the manipulations described in this study applied to a standardized tissue engineered construct, improved chondrogenesis to a measurable extent. As shown in FIGS. 23, 24A and 24B, in each case, the thickness of the

differentiated layer increased significantly, while cell viability, ECM production and expression of cartilage markers, *e.g.*, type II collagen, GAG increased (not shown). Young's modulus was significantly increased compared to control, and at the light microscopy level, the homogeneity of the synthesized matrix increased as well. Most significantly, the combination of these three treatment protocols has allowed us to achieve full-thickness chondrogenesis (FIG. 25).

Discussion:

The creation of full-thickness cartilage disks based on human mesenchymal stem cells removes a significant obstacle to translating cartilage tissue engineering from the laboratory to the clinic. Future work will include efforts to scale these constructs up beyond their already significant size. Our results suggest that manipulation of the cellular component of the constructs will be a valuable complement to scaffold and bioreactor design optimization in pursuit of these goals.

An additional problem that plagues tissue engineering using human MSCs is a large donor-to-donor variability in chondrogenic potential and biosynthetic activity. Each of the manipulations described in this study greatly reduced, but did not entirely

Example 6

Assessment of Mass Transport Limitations During Cartilage Tissue Engineering

Introduction:

Substrate mass transport considerations are very important during the growth of tissue engineered products *in vitro*. Due to the lack of an intrinsic convective supply/removal system in tissue engineered cartilage constructs grown in a bioreactor, the supply/removal of a given substrate to an individual cell within the construct will be limited by diffusion and cellular consumption. In a bioreactor system, mass transfer resistance can occur in one or a combination of regions of the system. External to the construct, mass transfer is dependent on the fluid hydrodynamics in the reactor, and the net consumption/production of the substrate by the tissue. The boundary layer adjacent to the growing tissue is where most of the fluid resistance, as well as mass transfer resistance external to the tissue, resides. Finally, in the implant itself, consumption/production of the substrate dominates. Mass transfer is primarily due to

passive diffusion, and a progressive increase in mass transfer resistance is expected as porosity decreases because of matrix production by the cells.

In mesenchymal stem cells (MSC)-based tissue engineered cartilage, some of the substrates required to induce chondrogenic differentiation are of higher molecular weight (*e.g.* TGF-β1, 25kDa), and have small diffusivities (10^{-7} cm^2/sec). The constructs initially contain metabolically active cells at very high density and, as the construct matures, abundant extracellular matrix (ECM) is produced. One would therefore predict that the penetration of substrates into the construct would be impeded by the molecular weight of the substrate but also by dynamic variables like the degree of maturity of the ECM and consumption rates by the cells. This hypothesis can explain the formation of a cartilaginous shell at the periphery of the construct (FIG. 28), with limited cell function at the center. The purpose of this study was to test this hypothesis by measuring mass transfer limitations of probe molecules within the tissue engineered construct as a function of its maturation state.

Methods:

Constructs:

Human bone marrow derived MSCs were isolated, culture expanded, and vacuum seeded onto 7x3 mm hyaluronan-based scaffolds (Hyaff-11®, Fidia Advanced Biopolymers, Abano Terme, Italy), as described previously. The assembled constructs were grown for up to 3 weeks in a continuous perfusion bioreactor with highly 0_2 permeable walls, in a defined chondrogenic medium (DMEM-HG supplemented with 1% ITS+Premix™, 100 μM ascorbate-2-phosphate, 10^{-7} M dexamethasone and 10 ng/ml TGF-β1).

Diffusion measurements:

To evaluate mass transport limitations of large molecules (*e.g.*, TGF-β), tissue engineered cartilage constructs were pulsed for 18 hours with fluorescently tagged dextran probes (Molecular Probes, Eugene, OR) of 3, 10, or 70 kDa molecular weight after 0 to 21 days in bioreactor culture. The probes were then cross-linked *in situ* using formalin, and paraffin sections were obtained. Composite digital images of the sections were used to obtain the local concentrations of the tracers using custom-written image

analysis routines (FIGS. 26A and 26B). A numerical model of diffusion into the constructs was developed.

Mass transport modeling:

To evaluate mass transport limitations of small molecules (*e.g.*, O_2), we developed a convection-diffusion model of mass transport in the reactor system with Michaelis-Menten consumption kinetics occurring in the construct. By using published consumption rates for the cells, the known physical and geometric characteristics of the bioreactor/construct system, and numerical methods, the model was solved to obtain oxygen concentration profiles for a variety of conditions such as the presence and the absence of an internal mixing system.

Results:

In FIGS. 26A and 26B, composition fluorescent micrographs illustrate the diffusion of fluorescent dextran probes into the constructs at several time points after the initiation of chondrogenic differentiation. Examples of the quantitative evaluation are shown in FIG. 27. As hypothesized, the depth of penetration of each probe decreased as a function of the MW of the probe and of the maturity of the construct.

Internal mixing of the bioreactor content at 8 ml/min has a profound effect on the 0_2 tension in the constructs. Steady-state O_2 concentration contours modeled in the bioreactor with (A) and without (B) internal mixing are shown in FIG. 28. The dashed line represents the interface between the cartilage and the medium. Under unmixed conditions, only the very surface of the construct is an O_2 tension of 0.2x atmospheric or better, with the center of the construct predicted to be nearly anoxic. (C) Partially differentiated construct grown under condition (B), showing the cartilaginous shell. The mixed model predicts that at 8 ml/min the bulk of the volume of the implant would be above 0.2x atmospheric O_2. This is nearly 2x higher than measured O_2 tensions in native cartilage samples.

Discussion:

These results suggest a central limiting role for substrate mass transport in the generation of large-scale cartilage implants. The results further support the hypothesis that both the MW of the substrate and the time elapsed since the beginning of

chondrogenic differentiation conspire to limit the diffusion of nutrients into the constructs.

Engineering approaches can improve mass transport across each region of the bioreactor/implant system; these include content mixing, changing the medium replenishment rates, and improving the hydrodynamics of the chamber to minimize boundary layer formation. The results from this study will be used to explore engineering solutions to the problem of shell formation, and to assess the countermeasures taken.

Cell preconditioning experiments:

Cells were transiently cultured in aggregate culture, then released, and seeded onto the scaffolds. All aggregate culture modes resulted in an improvement in cell viability and differentiation at the center of the constructs. Evaluation of aggregate culture times of 3, 5, and 7 days, followed by enzymatic release of the cells from the aggregates and assembly into the final constructs suggests that maintaining the cells in aggregate culture for 5 days (FIG. 29A) yields superior results to the 3 days (FIG. 29B) proposed earlier. This is in terms of implant viability, of the amount and "quality" of the synthesized matrix, and of the mechanical properties of the constructs (*e.g.* 30-50% increase in Young's modulus in "5 day aggregate" *vs.* "3 day aggregate" constructs). For example, the lighter areas in 3 day constructs (panel A, inset) although clearly viable are filled with a much less dense and organized chondroid matrix than equivalent areas in the 5 day constructs (panel B, inset). Our working hypothesis is that the pre-differentiation in aggregate culture lowers the metabolic requirements of the cells, thus pre-adapting them to the less favorable mass transport conditions in the larger constructs. Although the data are not yet conclusive, it is not clear that extending aggregate dwell time to 7 days results in further improvement (not shown). Taken together, these results, although preliminary, suggest that this preconditioning approach will be extremely useful for MSC-based cartilage tissue engineering.

What has been described above includes examples and implementations of the present invention. Because it is not possible to describe every conceivable combination of components, circuitry or methodologies for purposes of describing the present invention, one of ordinary skill in the art will recognize that many further combinations

and permutations of the present invention are possible. Accordingly, the present invention is intended to embrace all such alterations, modifications and variations that fall within the spirit and scope of the appended claims.

Having described the invention, the following is claimed.

1. A bioreactor system comprsing:

a chamber with an inlet port and an outlet port, the inlet port and outlet port allowing the chamber to be continuously perfused with a culture medium to facilitate the mass transfer rate of cells cultured the chamber.

2. A multi-channel fiber-optic spectrophotometer for measuring light transmission and absorbance of at least one molecular species in at least one sample, comprising:

a monochromator with an output slit plane;

a plurality of output fibers with polished ends, the polished ends of the fibers being positioned in the output slit plane;

a means for adjusting the wavelength of the monochomator under the control of a purpose written software; and

a means for automatically collecting, normalizing, and processing absorption and transmission data.

3. A method of improving chondrogenesis by mesenchymal stem cells, the method comprising the steps of:

providing a suspension of mesenchymal stem cells in a culture medium contained in a sterile vessel;

aggregating the cells in the vessel,

maintaining the aggregated cells in culture for a duration of time sufficient to allow chondrogenesis to begin;

releasing the cells from aggregate; and

seeding at least one construct with the released cells.

4. A pretreatment regimen to improve chondrogenesis by bone marrow derived mesenchymal stem cells, the pretreatment regimen comprising;

isolating bone marrow derived mesenchymal stem cells from bone marrow biopsies;

expanding the isolated bone marrow derived mesenchymal stem cells in culture; and

adding human recombinant fibroblast growth factor 2 (rhFGF-2) to the human bone marrow derived mesenchymal stem cell culture during culturing.

5. A treatment regimen to improve chondrogenesis by adult-bone marrow derived mesenchymal progenitor cells, the treatment regimen comprising:

providing bone marrow derived mesenchymal stem cells in a culture medium; the culture medium containing a first concentration of dexamethasone; and

reducing the concentration of dexamethasone in the culture medium during culturing to a second concentration substantially less than the first concentration, the second concentration of the dexmethasone being effective to induce the expression of BMP-2 in the cultured cells.

ABSTRACT

A bioreactor system includes a chamber with an inlet port and an outlet port. The inlet and outlet ports allow the chamber to be continuously perfused with a culture medium.

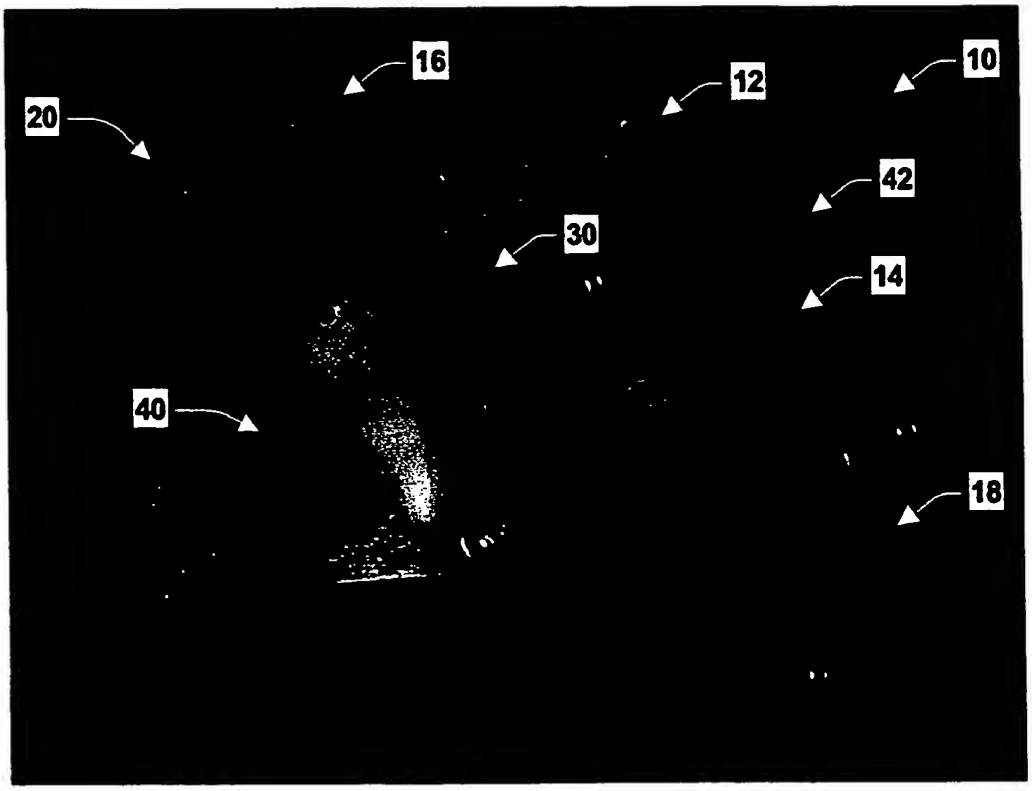

FIG. 1

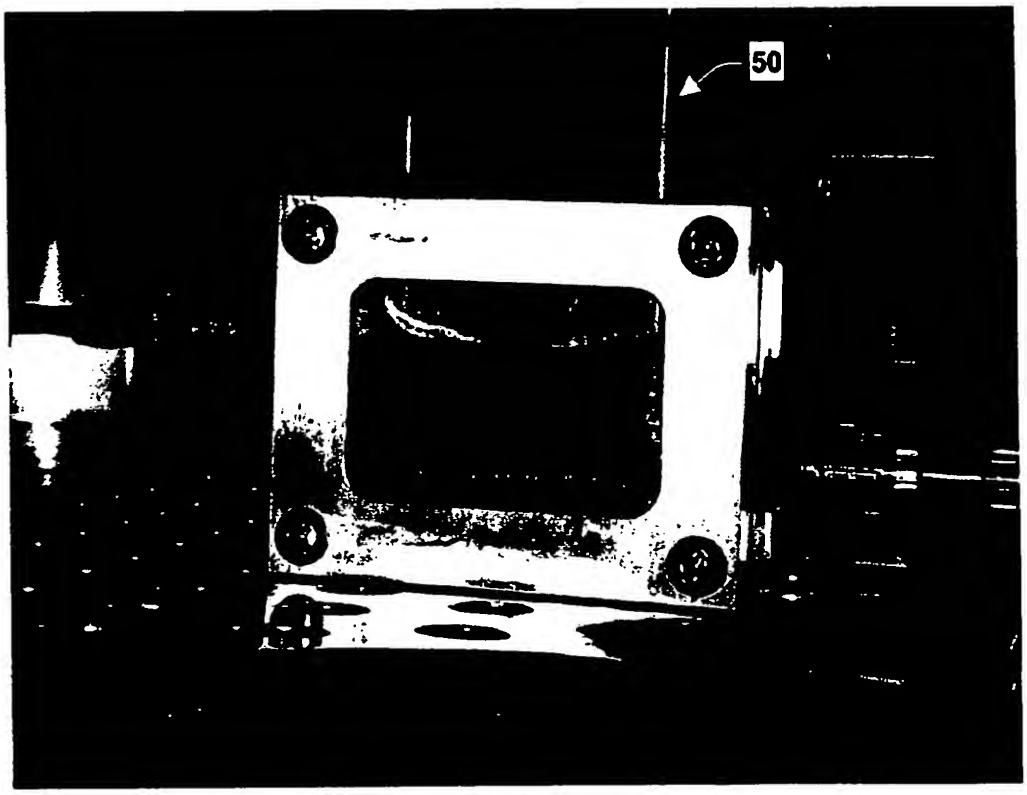

FIG. 2

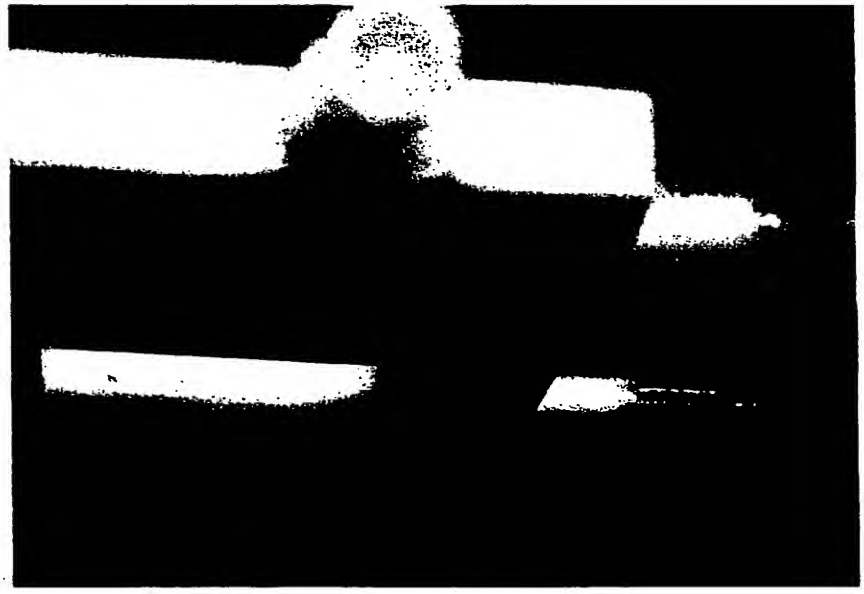

FIG. 3

FIG. 4

FIG. 5

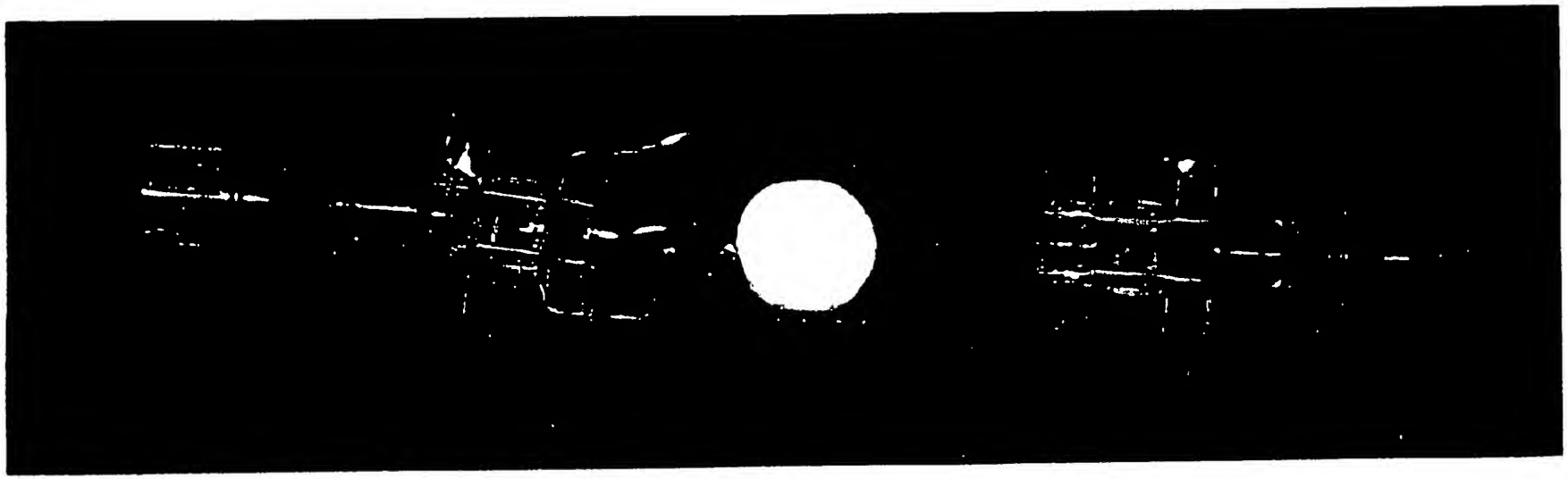

FIG. 6

FIG. 7

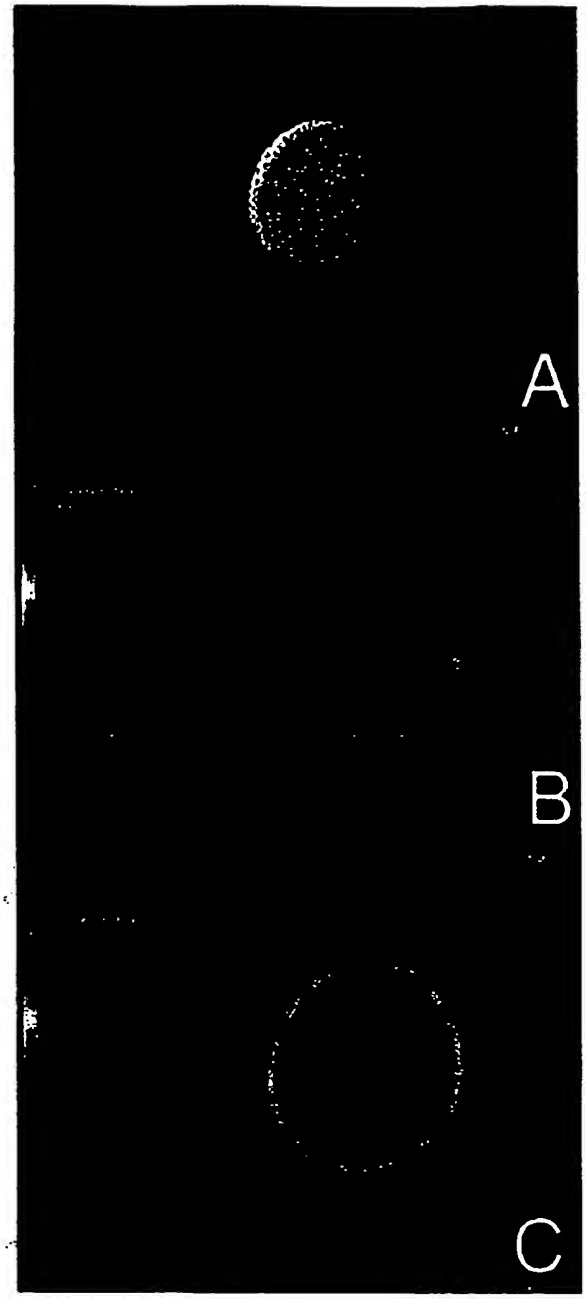

FIG. 8

FIG. 9

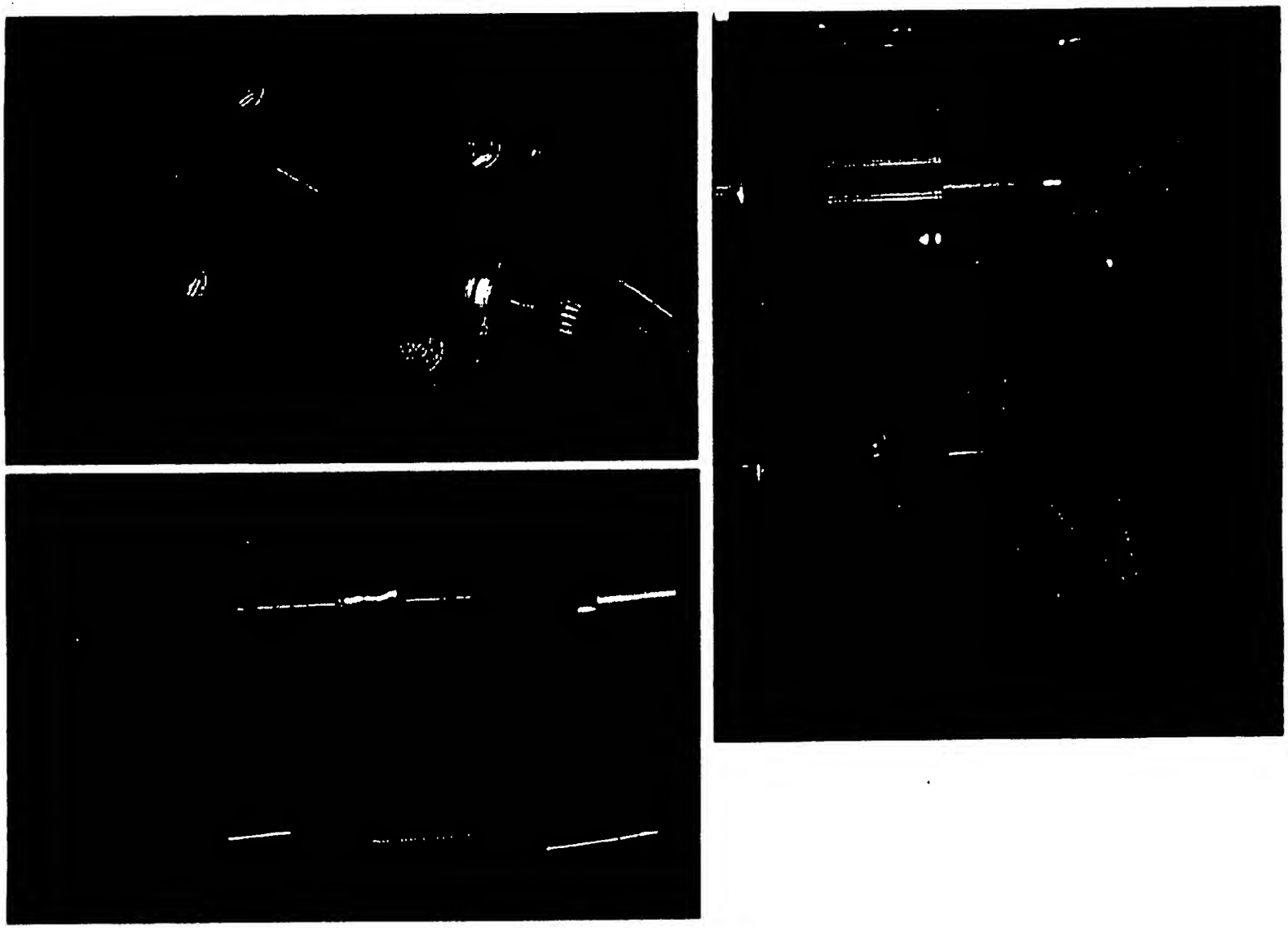

FIG. 10

FIG. 11

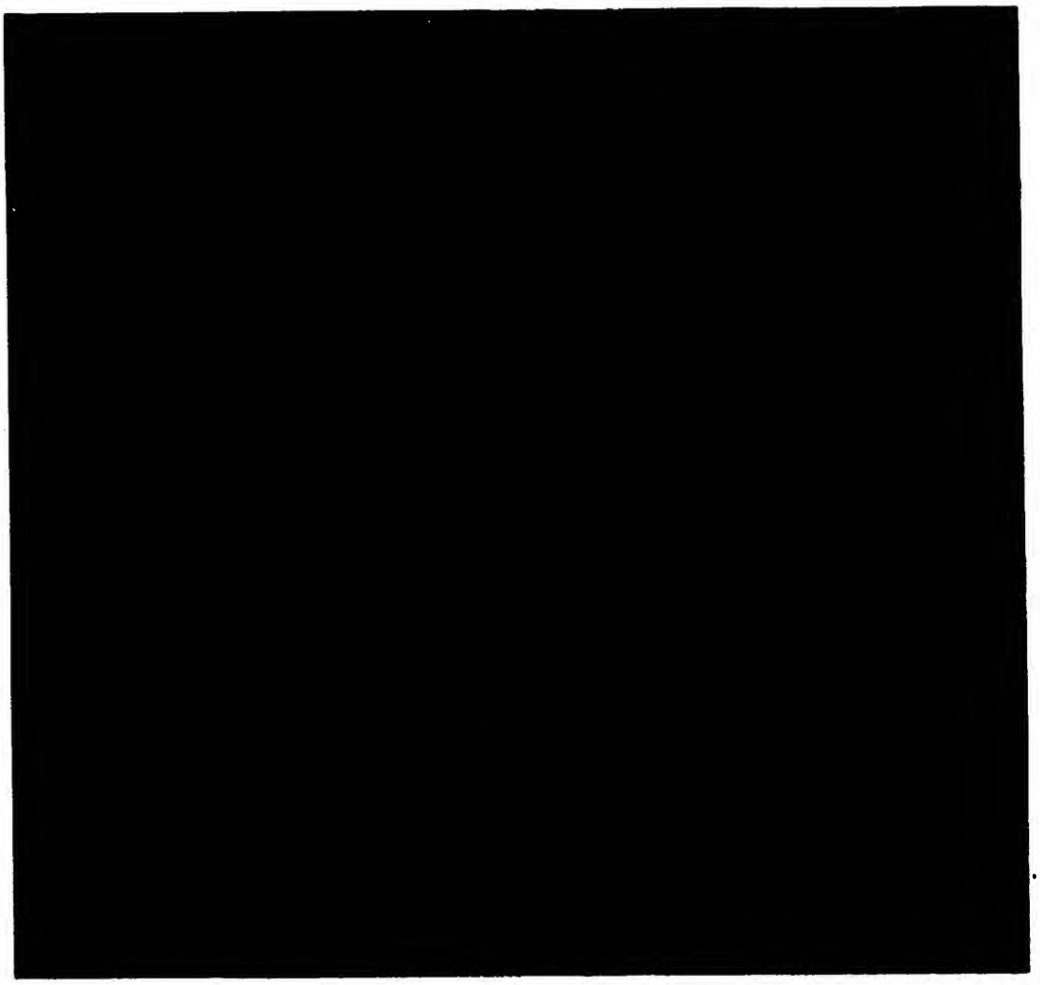

FIG. 12

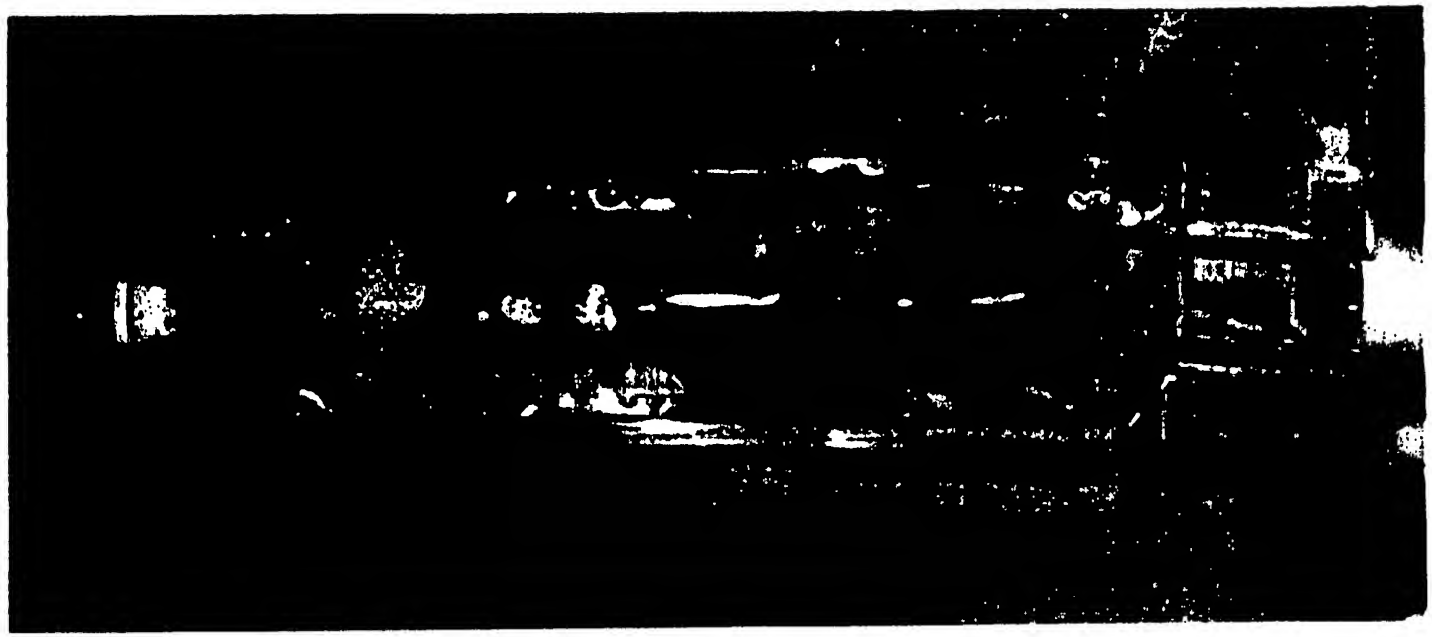

FIG. 13

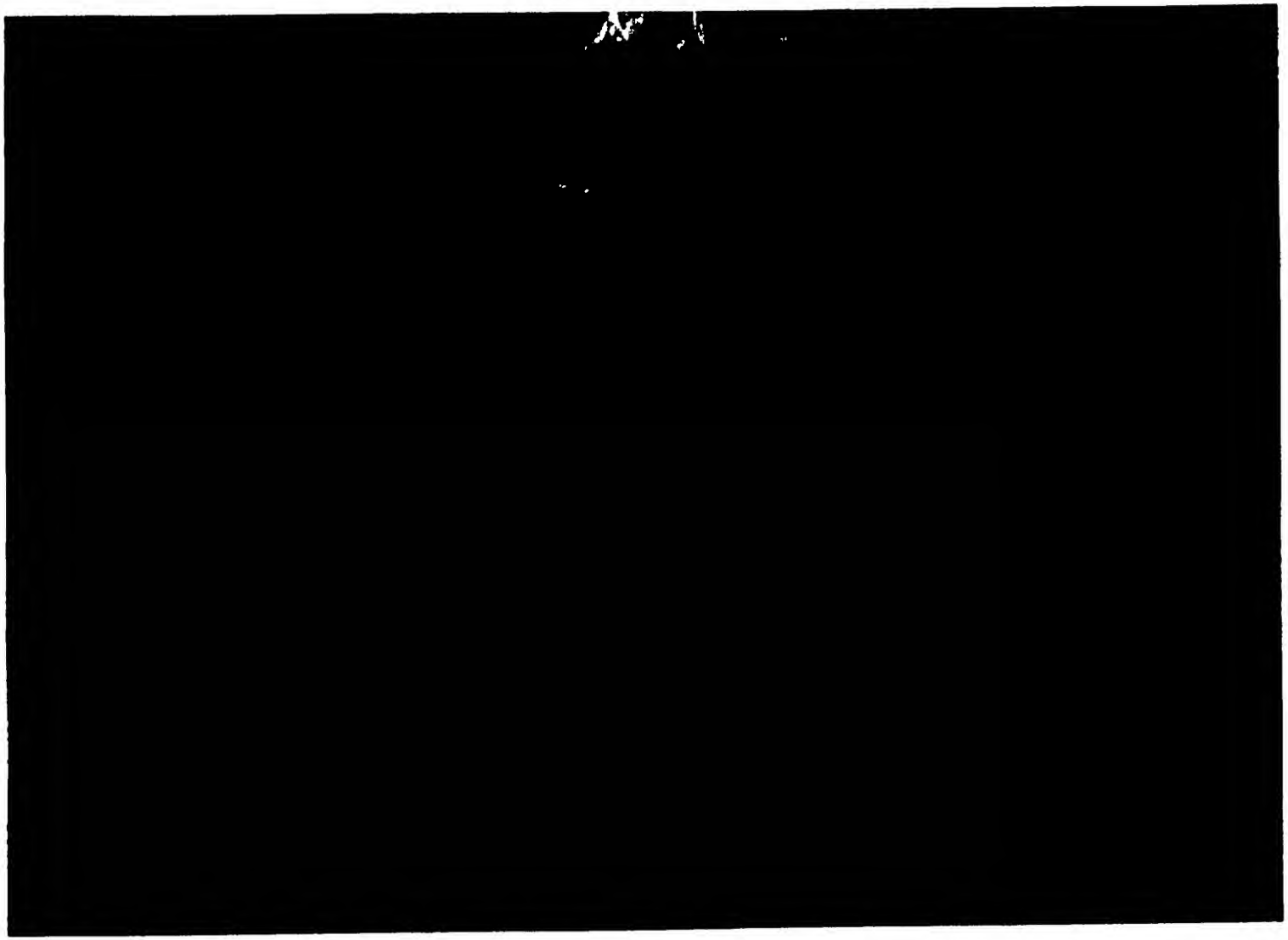

FIG. 14

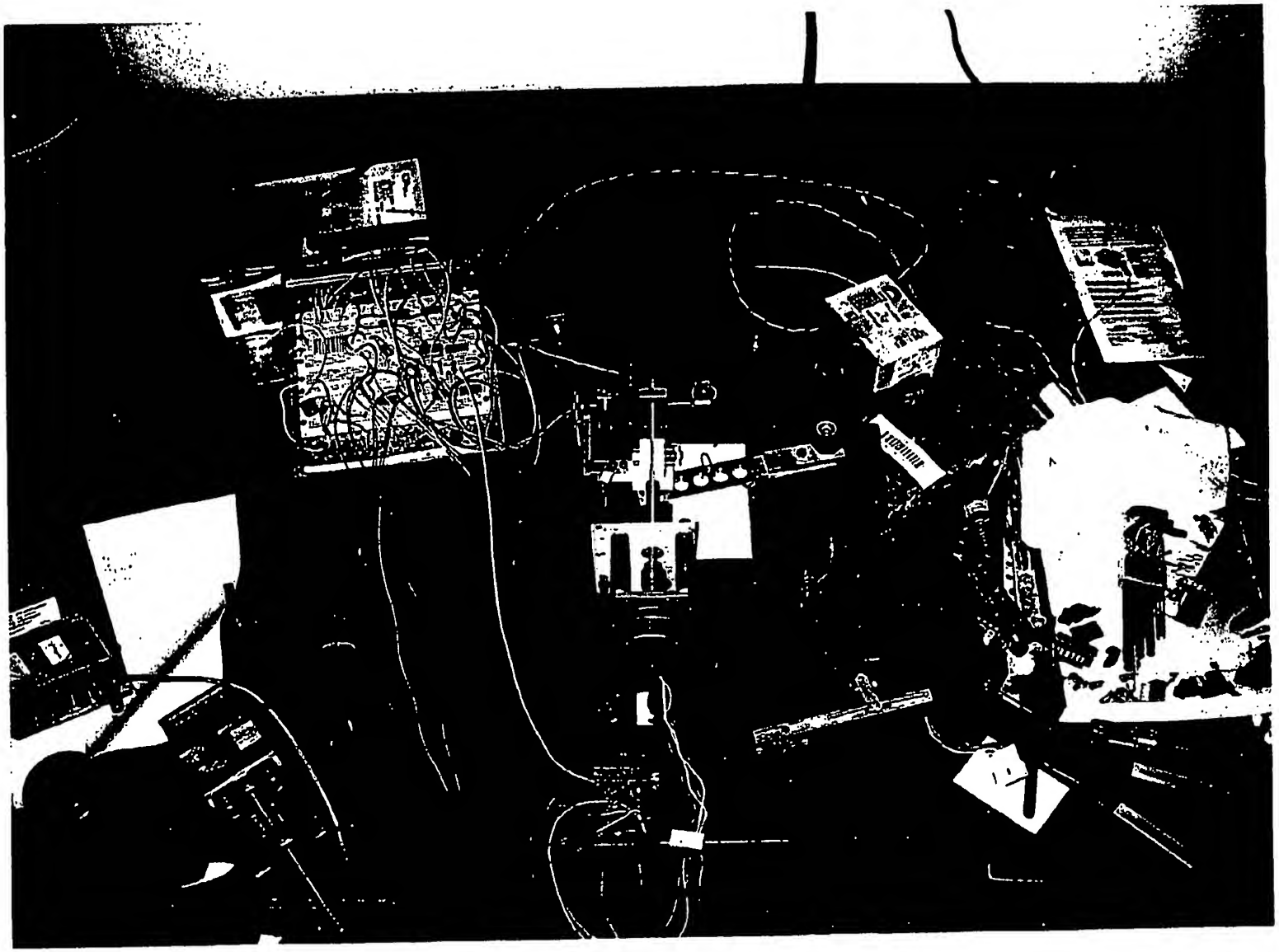

FIG. 15

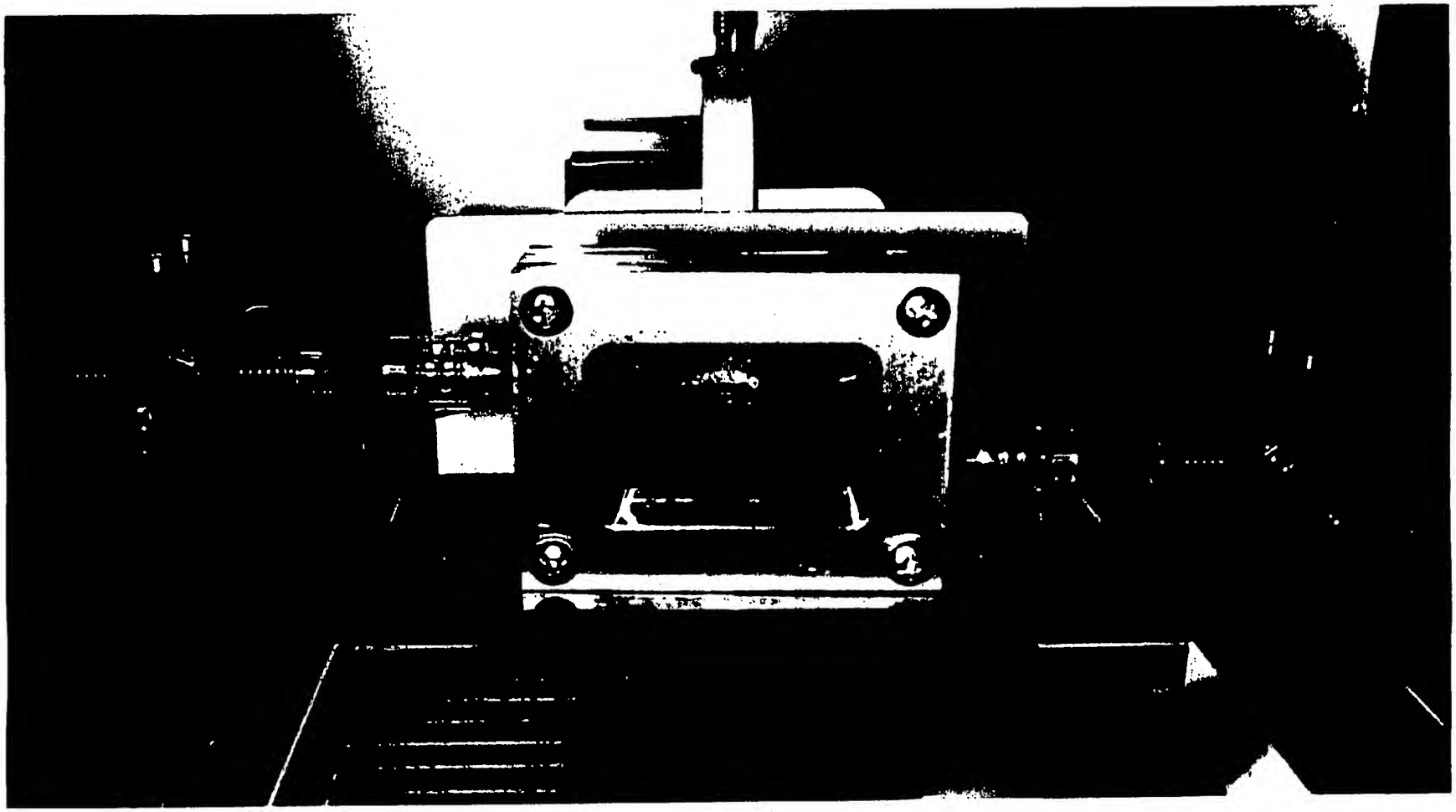

FIG. 16

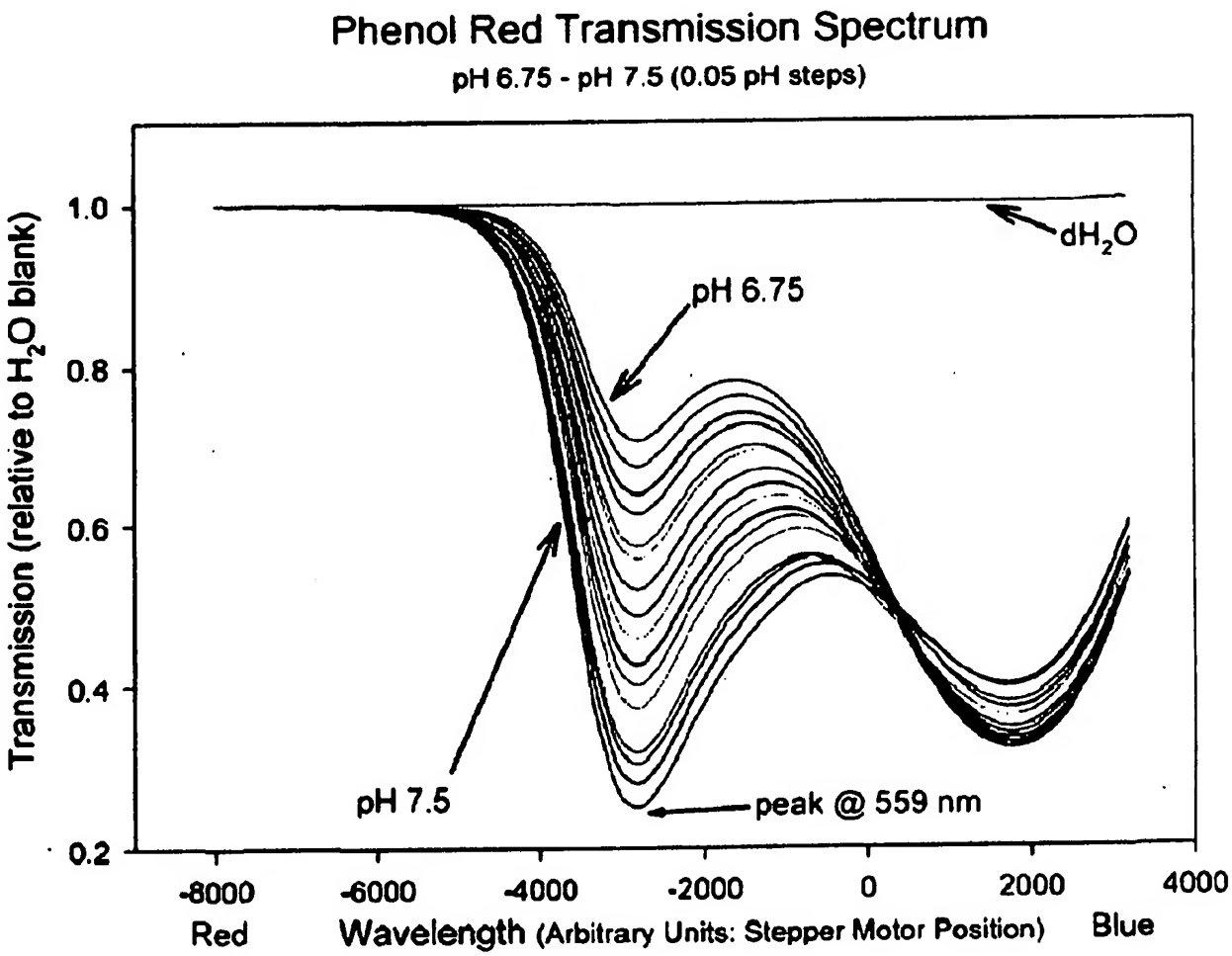

FIG. 17

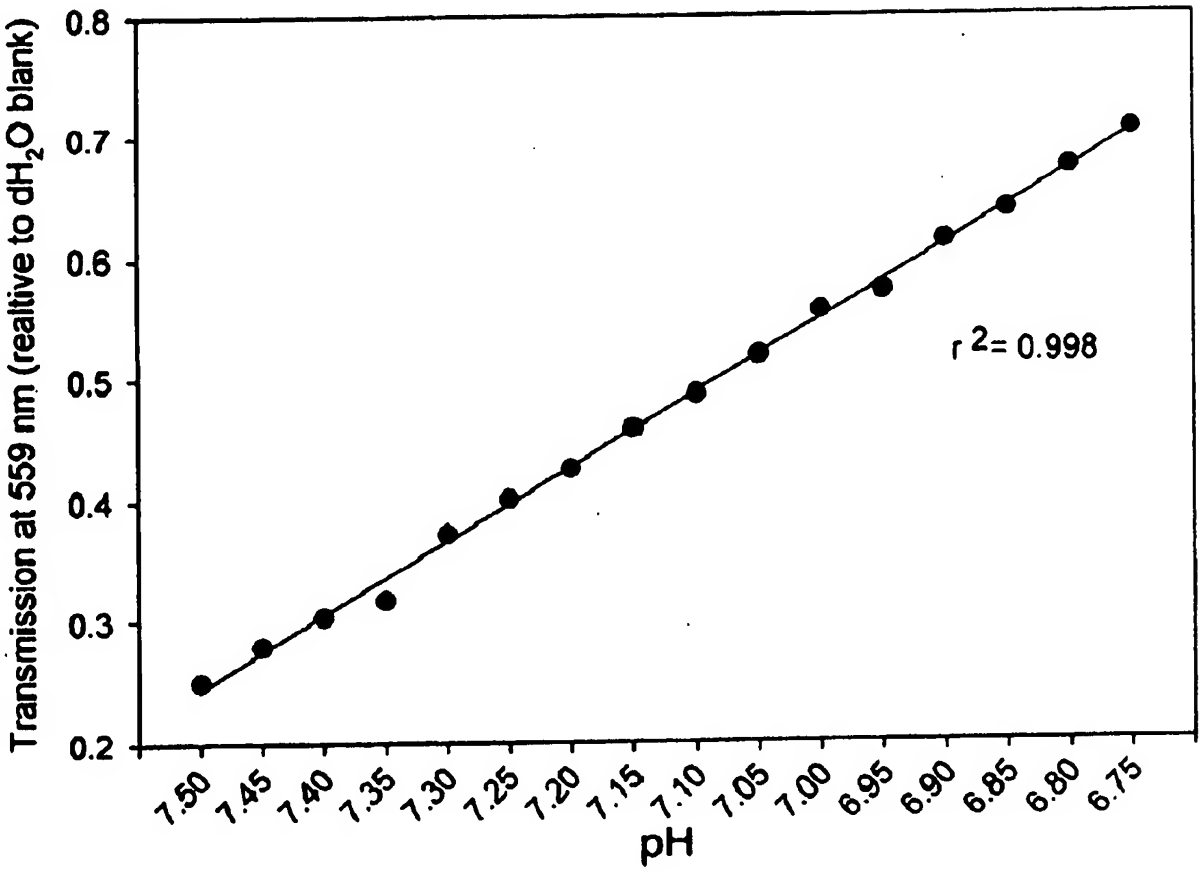

FIG. 18

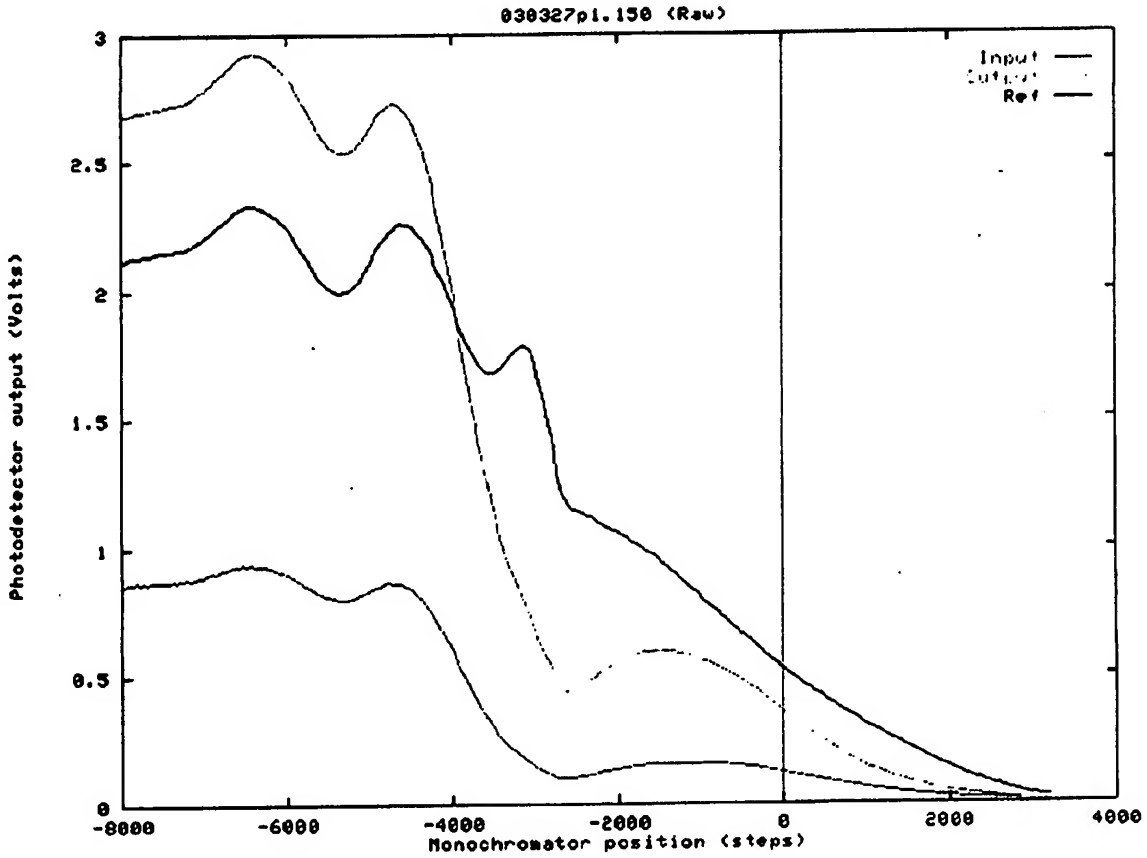

FIG. 19

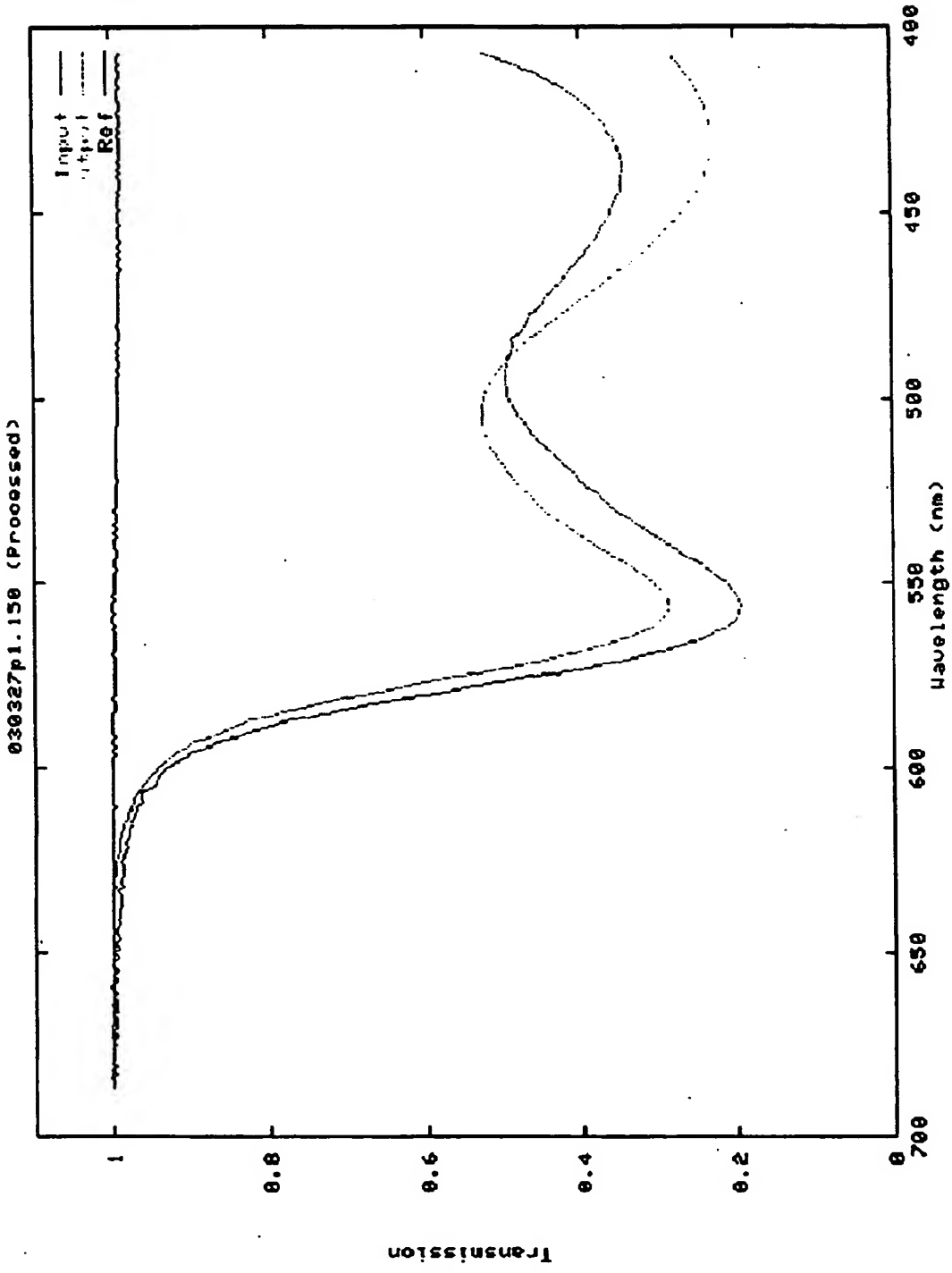

FIG. 20

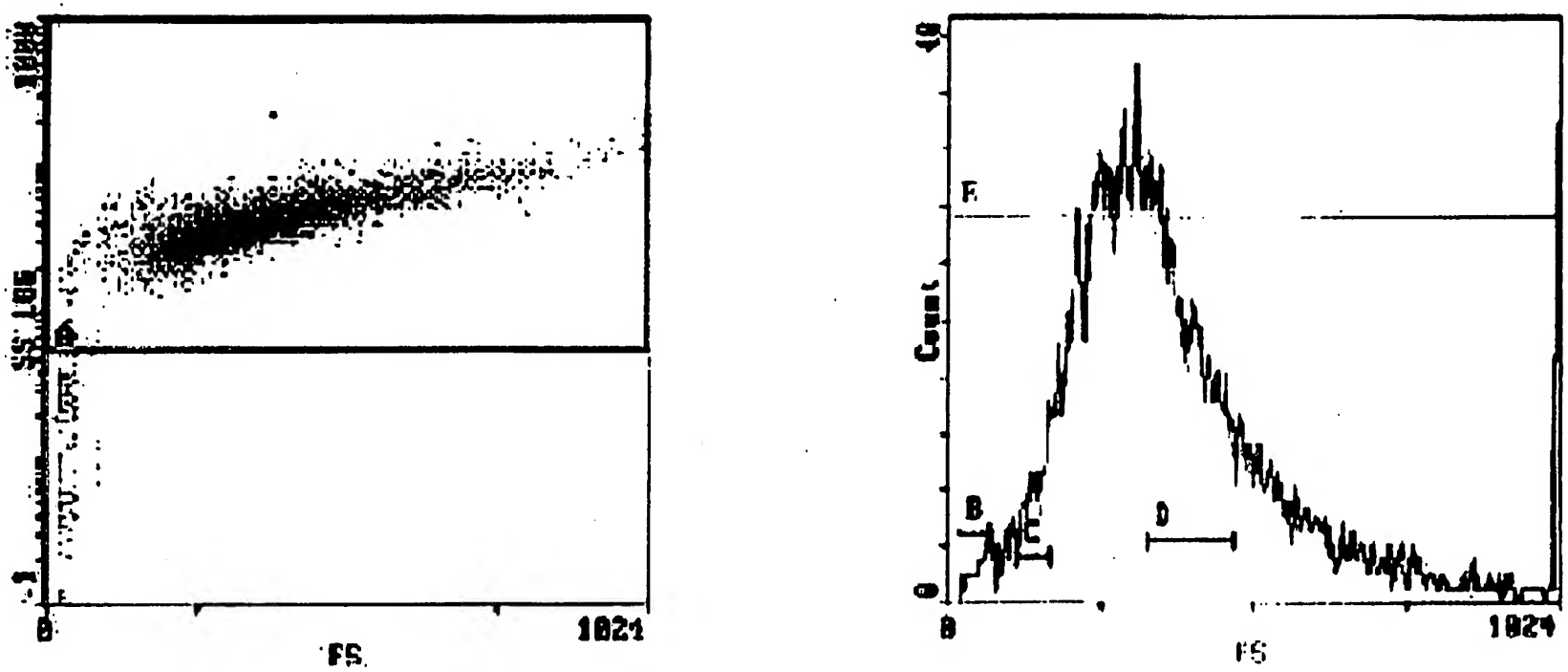

FIG. 21A

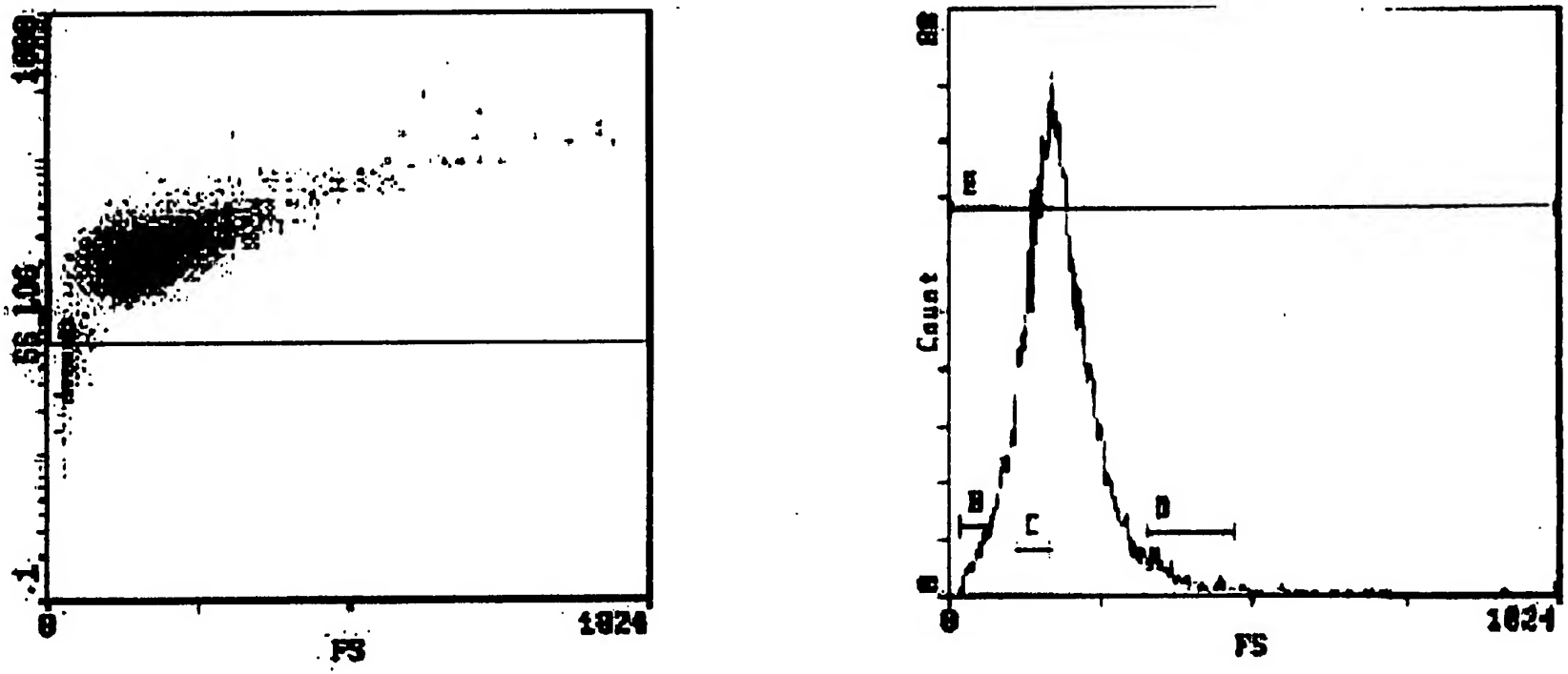

FIG. 21B

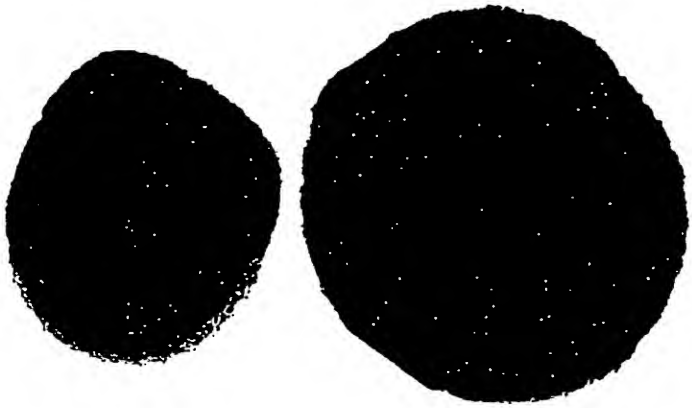

FIG. 22

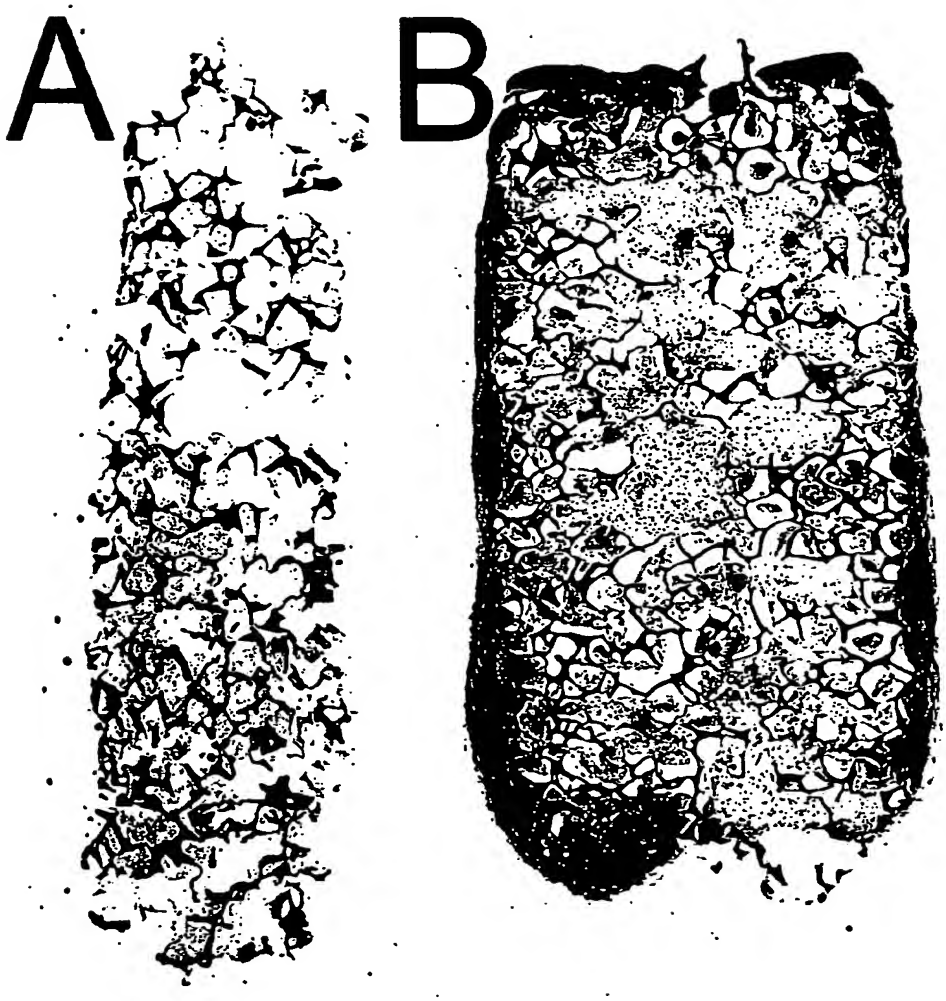

FIG. 23

FIG. 24A

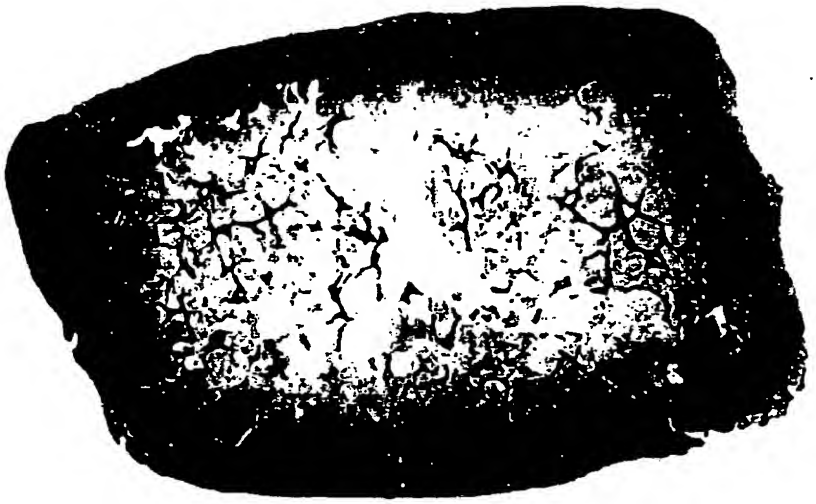

FIG. 24B

FIG. 25

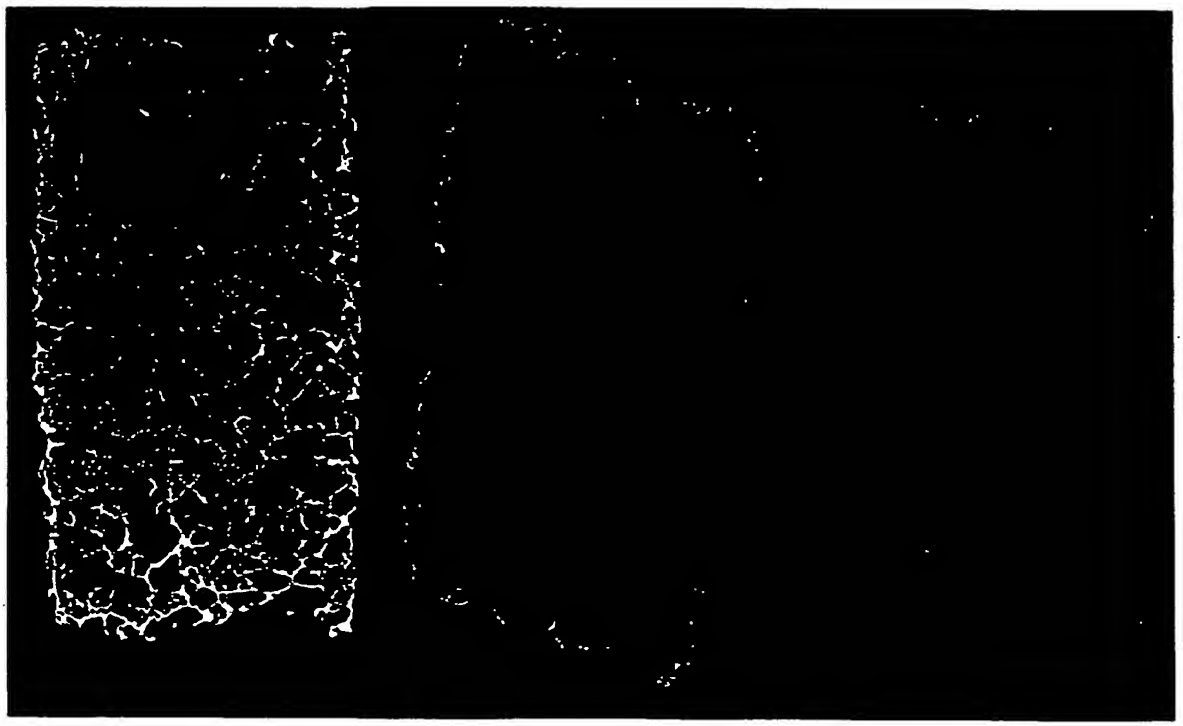

FIG. 26A

FIG. 26B

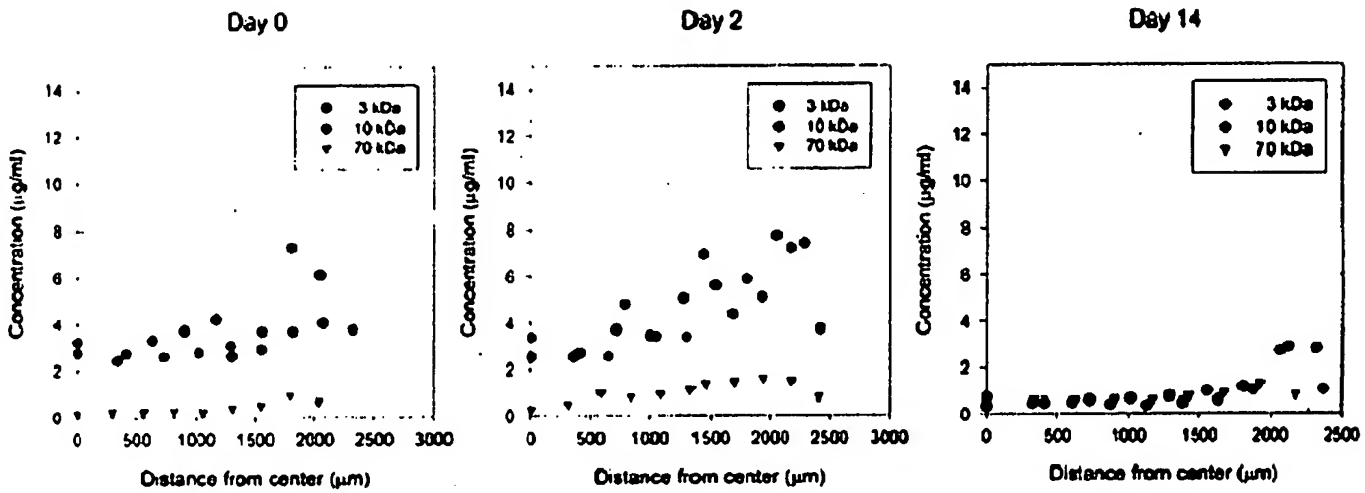

FIG. 27

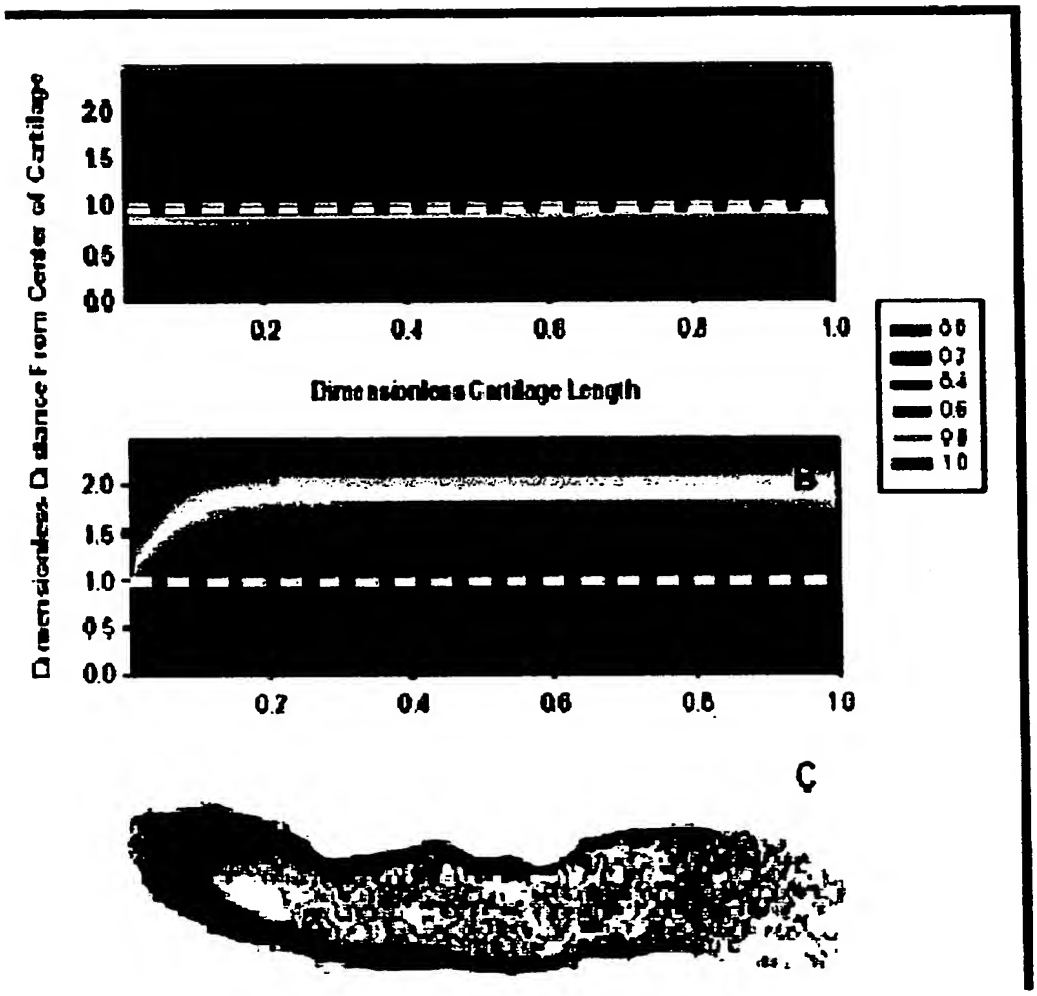

FIG. 28

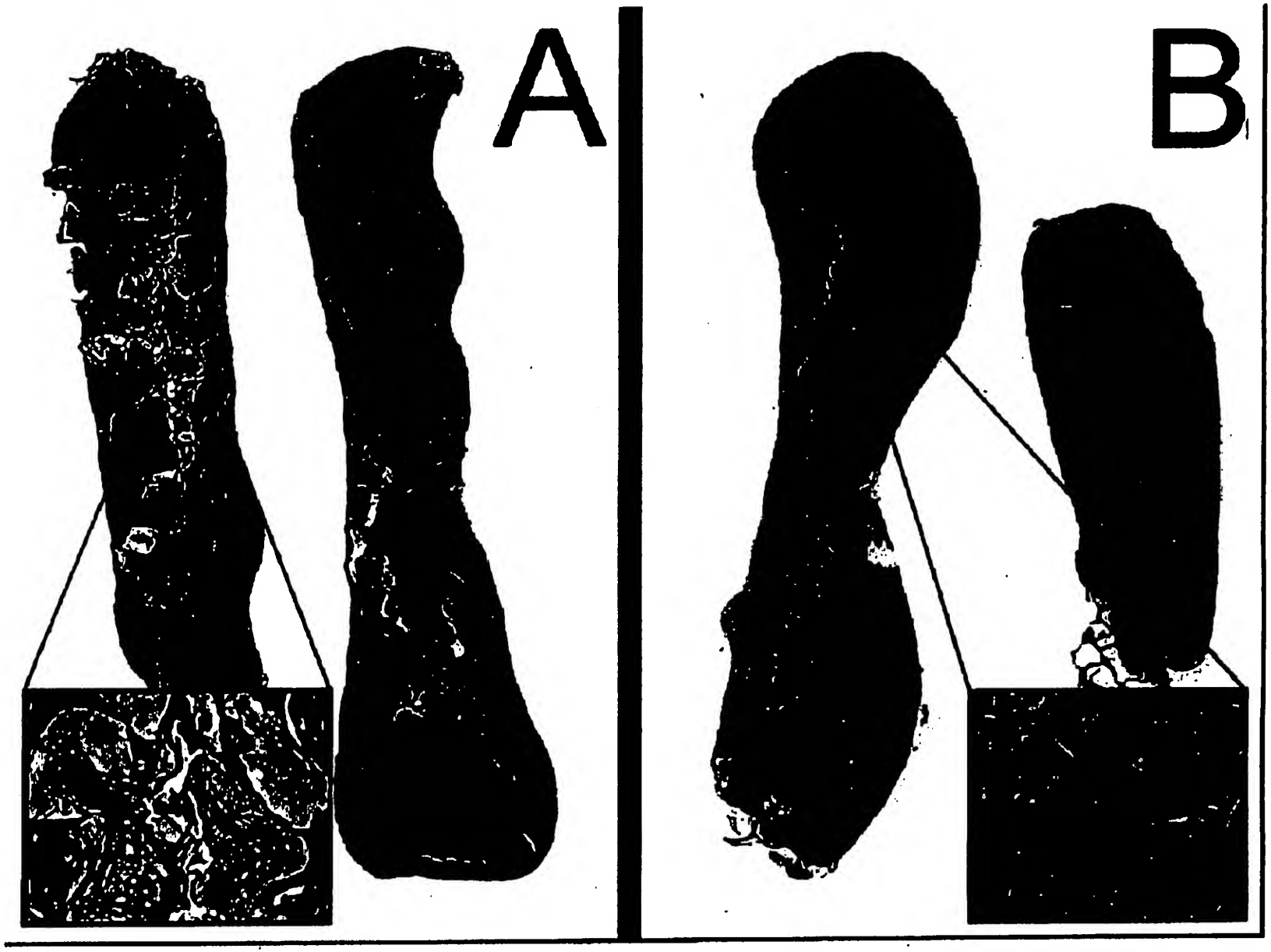

FIG. 29

Document made available under the Patent Cooperation Treaty (PCT)

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International filing date: 04 November 2004 (04.11.2004)

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